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STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

ATTORNEYS AT LAW

SUITE 600

1100 NEW YORK AVENUE, N.W.

WASHINGTON, D.C. 20005-3934

(202) 371-2600

FACSIMILE: (202) 371-2540



ROBERT GREENE
EDWARD J. KESSLER
JORGE A. GOLDSTEIN
SAMUEL L. FOX
DAVID K.S. CORNWELL
ROBERT W. ESMOND
TRACY-GENE G. DURKIN
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KAREN R. MARKOWICZ**
KIMBERLIN M. TOOHEY**

*BAR OTHER THAN D.C.
**REGISTERED PATENT AGENTS

WRITER'S DIRECT NUMBER:

(202) 789-5524

INTERNET ADDRESS:

lkerber@skgf.com

March 11, 1997

Box: New Patent Application

Assistant Commissioner for Patents
Washington, D.C. 20231

Re: U.S. Non-Provisional Utility Patent Application
Appl. No. To be assigned; Filed: March 11, 1997
For: **Method for the Identification of Agents for Use in the Treatment of
Alzheimer's Disease**
Inventors: Atwood *et al.*
Our Ref: 0609.4350000/REF/LLK

Sir:

The following documents are forwarded herewith for appropriate action by the U.S.
Patent and Trademark Office:

1. U.S. Non-Provisional Utility Patent Application entitled:

Method for the Identification of Agents for Use in the Treatment of Alzheimer's
Disease

and naming as inventors:

100 ET AL
Craig S. Atwood
Ashley I. Bush
Xudong Huang, and
Rudolph E. Tanzi

Assistant Commissioner for Patents
March 11, 1997
Page 2

the application comprising:

a. A specification containing:

- (i) 49 pages of description prior to the claims;
- (ii) 9 pages of claims (39 claims);
- (iii) a one (1) page abstract;

b. 17 sheets of drawings: (Figures 1-12); and

2. Two (2) return postcards.

It is respectfully requested that, of the two attached postcards, one be stamped with the filing date of these documents and returned to our courier, and the other, prepaid postcard, be stamped with the filing date and unofficial application number and returned as soon as possible.

This patent application is being submitted under 37 C.F.R. § 1.53(b)(1) without Declaration and without filing fee.

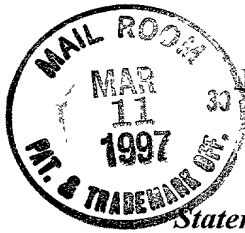
Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.



Lori L. Kerber
Attorney for Applicants
Registration No. P-41,113

REF/LLK:ttp
Enclosures



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Method for the Identification of Agents for Use in the Treatment of Alzheimer's Disease

Statement as to Rights to Inventions Made Under Federally-Sponsored Research and Development

5 Part of the work performed during the development of this invention utilized U.S. Government Funds under Grant No. R29AG12686 from the National Institutes of Health. The government may have certain rights in this invention.

Background of the Invention

10 Polymers of Abeta (A β), the 4.3 kD, 39-43 amino acid peptide product of the transmembrane protein, amyloid protein precursor (APP), are the main components extracted from the neuritic and vascular amyloid of Alzheimer's disease (AD) brains. A β deposits are usually most concentrated in regions of high neuronal cell death, and may be present in various morphologies, including
15 amorphous deposits, neurophil plaque amyloid, and amyloid congophilic angiopathy (Masters, C.L., *et al.*, *EMBO J.* 4:2757 (1985); Masters, C.L. *et al.*, *Proc. Natl. Acad. Sci. USA* 82: 4245 (1985)). Growing evidence suggests that amyloid deposits are intimately associated with the neuronal demise that leads to dementia in the disorder.

20 The presence of an enrichment of the 42 residue species of A β in these deposits suggests that this species is more pathogenic. The 42 residue form of A β (A β ₁₋₄₂), while a minor component of biological fluids, is highly enriched in amyloid, and genetic studies strongly implicate this protein in the etiopathogenesis of AD. Amyloid deposits are decorated with inflammatory
25 response proteins, but biochemical markers of severe oxidative stress such as peroxidation adducts, advanced glycation end-products, and protein cross-linking are seen in proximity to the lesions. To date, the cause of A β deposits is unknown, although it is believed that preventing these deposits may be a means of treating the disorder.

When polymers of A β are placed into culture with rat hippocampal neurons, they are neurotoxic (Kuo, Y-M., *et al.*, *J. Biol. Chem.* 271:4077-81 (1996); Roher, A.E., *et al.*, *Journal of Biological Chemistry* 271:20631-20635 (1996)). The mechanism underlying the formation of these neurotoxic polymeric A β species remains unresolved. The overexpression of A β alone cannot sufficiently explain amyloid formation, since the concentration of A β required for precipitation is not physiologically plausible. That alterations in the neurochemical environment are required for amyloid formation is indicated by its solubility in neural phosphate buffer at concentrations of up to 16 mg/ml (Tomski, S. & Murphy, R.M. *Archives of Biochemistry and Biophysics* 294:630 (1992)), biological fluids such as cerebrospinal fluid (CSF) (Shoji, M., Golde *et al.* (1992); Seubert, P. (1992); Haass *et al.* (1992)) and in the plaque-free brains of Down's syndrome patients (Teller, J.K., *et al.*, *Nature Medicine* 2:93-95 (1996)).

Studies into the neurochemical vulnerability of A β to form amyloid have suggested altered zinc and [H⁺] homeostasis as the most likely explanations for amyloid deposition. A β is rapidly precipitated under mildly acidic conditions *in vitro* (pH 3.5-6.5) (Barrow C.J. & Zagorski M.G., *Science* 253:179-182 (1991); Fraser, P.E., *et al.*, *Biophys. J.* 60:1190-1201 (1991); Barrow, C.J., *et al.*, *J. Mol. Biol.* 225:1075-1093 (1992); Burdick, D., *J. Biol. Chem.* 267:546-554 (1992); Zagorski, M.G. and Barrow, C.J., *Biochemistry* 31:5621-5631 (1992); Kirshenbaum, K. and Daggett, V., *Biochemistry* 34:7629-7639 (1995); Wood, S.J., *et al.*, *J. Mol. Biol.* 256:870-877 (1996)). Recently, it has been shown that the presence of certain biometals, in particular redox inactive Zn²⁺ and, to a lesser extent, redox active Cu²⁺ and Fe²⁺, markedly increases the precipitation of soluble A β (A.T. Bush *et al.*, *J. Biol. Chem.* 268:16109 (1993); A.I. Bush *et al.*, *J. Biol. Chem.* 269:12152 (1994); A.I. Bush *et al.*, *Science* 265:1464 (1994); A.I. Bush, *et al.*, *Science* 268:1921 (1995)). At physiological pH, A β ₁₋₄₀ specifically and saturably binds Zn²⁺, manifesting high affinity binding (K_D = 107 nM) with a 1:1 (Zn²⁺:A β) stoichiometry, and low affinity binding (K_D = 5.2 μ M) with a 2:1

stoichiometry. However, the complete reversibility of Zn-induced A β ₁₋₄₀ aggregation in the presence of divalent metal ion chelating agents suggests that zinc binding is a reversible, normal function of A β and implicates other neurochemical mechanisms in the formation of amyloid. A process involving irreversible A β aggregation, such as the crosslinking of A β monomers, in the formation of polymeric species of A β that are present in amyloid plaques is thus a more plausible explanation for the formation of neurotoxic polymeric A β species.

The reduction by APP of copper (II) to copper (I) may lead to irreversible A β aggregation and crosslinking. This reaction may promote an environment that would enhance the production of hydroxyl radicals, which may contribute to oxidative stress in AD (Multhaup, G., *et al.*, *Science* 271:1406-1409 (1996)). A precedence for abnormal Cu metabolism already exists in the neurodegenerative disorders of Wilson's disease, Menkes' syndrome and possibly familial amyotrophic lateral sclerosis (Tanzi, R.E. *et al.*, *Nature Genetics* 5:344 (1993); Bull, P.C., *et al.*, *Nature Genetics* 5:327-x (1993); Vulpe, C., *et al.*, *Nature Genetics* 3:7 (1993); Yamaguchi, Y., *et al.*, *Biochem. Biophys. Res. Commun.* 197:271 (1993); Chelly, J. *et al.*, *Nature Genetics* 3:14 (1993); Wang, D. & Munoz, D.G., *J. Neuropathol. Exp. Neurol.* 54:548 (1995); Beckman, J.S., *et al.*, *Nature* 364:584 (1993); Hartmann, H.A. & Evenson, M.A., *Med. Hypotheses* 38:75 (1992)).

Although much fundamental pathology, genetic susceptibility and biology associated with AD is becoming clearer, a rational chemical and structural basis for developing effective drugs to prevent or cure the disease remains elusive. While the genetics of the disorder indicates that the metabolism of A β is intimately associated with the etiopathogenesis of the disease, drugs for the treatment of AD have so far focused on "cognition enhancers" which do not address the underlying disease processes.

Summary of the Invention

The invention relates to a method for the identification of an agent to be used in the treatment of AD, wherein the agent is capable of altering the production of Cu(I) by A β , the method comprising:

- 5 (a) adding Cu(II) to a first A β sample;
- (b) allowing the first sample to incubate for an amount of time sufficient to allow said first sample to generate Cu(I);
- (c) adding Cu(II) to a second A β sample, the second sample additionally comprising a candidate pharmacological agent;
- 10 (d) allowing the second sample to incubate for the same amount of time as the first sample;
- (e) determining the amount of Cu(I) produced by the first sample and the second sample; and
- (f) comparing the amount of Cu(I) produced by the first sample to the amount of Cu(I) produced by the second sample;
- 15 whereby a difference in the amount of Cu(I) produced by the first sample as compared to the second sample indicates that the candidate pharmacological agent has altered the production of Cu(I) by A β .

In a preferred embodiment, the amount of Cu(I) present in said first and said second sample is determined by

- 20 (a) adding a complexing agent to said first and said second sample, wherein said complexing agent is capable of combining with Cu(I) to form a complex compound, wherein said complex compound has an optimal visible absorption wavelength;
- 25 (b) measuring the absorbancy of said first and said second sample; and
- (c) calculating the concentration of Cu(I) in said first and said second sample using the absorbancy obtained in step (b).

In a more preferred embodiment, the complexing agent is bathocuproinedisulfonic (BC) anion. The concentration of Cu^+ produced by $\text{A}\beta$ may then be calculated on the basis of the absorbance of the sample at about 478 nm to about 488 nm, more preferable about 480 to about 486 nm, and most preferably about 483 nm.

In another aspect, the invention relates to a method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of altering the production of Fe(II) by $\text{A}\beta$, said method comprising:

- (a) adding Fe(III) to a first $\text{A}\beta$ sample;
 - (b) allowing said first sample to incubate for an amount of time sufficient to allow said first sample to generate Cu^+ ;
 - (c) adding Fe(III) to a second $\text{A}\beta$ sample, said second sample additionally comprising a candidate pharmacological agent;
 - (d) allowing said second sample to incubate for the same amount of time as said first sample;
 - (e) determining the amount of Fe(II) produced by said first sample and said second sample; and
 - (f) comparing the amount of Fe(II) present in said first sample to the amount of Fe(II) present in said second sample;
- whereby a difference in the amount of Fe(II) present in said first sample as compared to said second sample indicates that said candidate pharmacological agent has altered the production of Fe(II) by $\text{A}\beta$.

In a preferred embodiment, the amount of Fe(II) present is determined by using a spectrophotometric method analogous to that used for the determination of Cu(I) , above. In this method, the complexing agent is batho-phenanthrolinedisulfonic (BP) anion. The concentration of Fe^{2+} -BP produced by $\text{A}\beta$ may then be calculated on the basis of the absorbance of the sample at about 530 to about 540 nm, more preferably about 533 nm to about 538 nm, and most preferably about 535 nm.

In yet another aspect, the invention relates to a method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of altering the production of H_2O_2 by $A\beta$, said method comprising:

- (a) adding Cu(II) or Fe(III) to a first $A\beta$ sample;
- 5 (b) allowing said first sample to incubate for an amount of time sufficient to allow said first sample to generate H_2O_2 ;
- (c) adding Cu(II) or Fe(III) to a second $A\beta$ sample, said second sample additionally comprising a candidate pharmacological agent;
- 10 (d) allowing said second sample to incubate for the same amount of time as said first sample;
- (e) determining the amount of H_2O_2 produced by said first sample and said second sample; and
- (f) comparing the amount of H_2O_2 present in said first sample to the amount of H_2O_2 present in said second sample;
- 15 whereby a difference in the amount of H_2O_2 present in said first sample as compared to said second sample indicates that said candidate pharmacological agent has altered the production of H_2O_2 by $A\beta$.

In a preferred embodiment, the determination of the amount of H_2O_2 present in said first and said second sample is determined by

- 20 (a) adding catalase to a first aliquot of said first sample obtained in step (a) of claim 1 in an amount sufficient to break down all of the H_2O_2 generated by said sample;
- (b) adding TCEP, in an amount sufficient to capture all of the H_2O_2 present in said samples, to
- 25 (i) said first aliquot
- (ii) a second aliquot of said first sample obtained in step (a) of claim 1; and
- (iii) said second sample obtained in step (b) of claim 1;
- (c) incubating the samples obtained in step (b) for an amount
- 30 of time sufficient to allow the TCEP to capture all of the H_2O_2 ;

- (d) adding DTNB to said samples obtained in step (c);
- (e) incubating said samples obtained in step (d) for an amount of time sufficient to generate TMB;
- (f) measuring the absorbancy at about 407 to about 417 nm of said samples obtained in step (e); and
- (g) calculating the concentration of H_2O_2 in said first and said second sample using the absorbancies obtained in step (f). In a preferred embodiment, the absorbancy of TMB is measured at about 412 nm.

In another aspect, the invention relates to a method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of decreasing the production of O_2^- by $A\beta$, said method comprising:

- (a) adding $A\beta$ and to a first buffer sample having an O_2 tension greater than 0;
 - (b) allowing said first sample to incubate for an amount of time sufficient to allow said first sample to generate O_2^- ;
 - (c) adding $A\beta$ and a candidate pharmacological agent to a second buffer sample having an O_2 tension greater than 0;
 - (d) allowing said second sample to incubate for the same amount of time as said first sample;
 - (e) determining the amount of O_2^- produced by said first sample and said second sample; and
 - (f) comparing the amount of O_2^- present in said first sample to the amount of O_2^- present in said second sample;
- whereby a difference in the amount of O_2^- present in said first sample as compared to said second sample indicates that said candidate pharmacological agent has altered the production of O_2^- by $A\beta$. In a preferred embodiment, the $A\beta$ used is $A\beta_{1-42}$.

Because the ability of $A\beta$ to generate H_2O_2 from O_2^- may in many instances be beneficial, in a preferred embodiment, the invention also relates to a method for the identification of an agent to be used in the treatment of AD,

wherein said agent is capable of interfering with the interaction of O_2 and $A\beta$ to produce O_2^- , without interfering with the SOD-like activity of $A\beta$, said method comprising:

(a) identifying an agent capable of decreasing the production of O_2^- by $A\beta$; and

(b) determining the ability of said agent to alter the SOD-like activity of $A\beta$. In a preferred embodiment, the determination of the ability of said agent to alter the SOD-like activity of $A\beta$ is made by determining whether $A\beta$ is capable of catalytically producing $Cu(I)$, $Fe(II)$ or H_2O_2 .

Brief Description of the Figures

Figure 1 is a graph showing the proportion of soluble $A\beta_{1-40}$ remaining following centrifugation of reaction mixtures.

Figures 2A, 2B and 2C: Figure 2A is a graph showing the proportion of soluble $A\beta_{1-40}$ remaining in the supernatant after incubation with various metal ions. Figure 2B is a graph showing a turbidometric analysis of pH effect on metal ion-induced $A\beta_{1-40}$ aggregation. Figure 2C is a graph showing the proportion of soluble $A\beta_{1-40}$ remaining in the supernatant after incubation with various metal ions, where high metal ion concentrations were used.

Figure 3 is a graph showing a competition analysis of $A\beta_{1-40}$ binding to Cu^{2+} .

Figures 4A, 4B and 4C: Figure 4A is a graph showing the proportion of soluble $A\beta_{1-40}$ remaining in the supernatant following incubation at various pHs in $PBS \pm Zn^{2+}$. Figure 4B is a graph showing the proportion of soluble $A\beta_{1-40}$ remaining in the supernatant following incubation at various pHs with different Cu^{2+} concentrations. Figure 4C is a graph showing the relative aggregation of $A\beta_{1-40}$ at pH 7.4 and 6.6 with different Cu^{2+} concentrations.

Figures 5A and 5B: Figure 5A is a graph showing a turbidometric analysis of Cu^{2+} -induced $A\beta_{1-40}$ aggregation at pH 7.4 reversed by successive cycles of

chelator. Figure 5B is a graph showing a turbidometric analysis of the reversibility of Cu^{2+} -induced $\text{A}\beta_{1-40}$ aggregation as the pH cycles between 7.4 and 6.6.

Figure 6 shows the amino acid sequence of $\text{APP}_{669-716}$ near $\text{A}\beta_{1-42}$. Rat $\text{A}\beta$ is mutated (R5G, Y10F, H13R; bold). Possible metal-binding residues are underlined.

Figure 7 is a graph showing the effects of pH, Zn^{2+} or Cu^{2+} upon $\text{A}\beta$ formation.

Figure 8 is a western blot showing the extraction of $\text{A}\beta$ from post-mortem brain tissue.

Figure 9 is a western blot showing $\text{A}\beta$ crosslinking by copper.

Figure 10 is a graph showing Cu(I) generation by $\text{A}\beta$.

Figure 11 is a graph showing H_2O_2 production by $\text{A}\beta$.

Figure 12 is a model for free radical and amyloid formation in Alzheimer's disease.

Detailed Description of the Preferred Embodiments

Definitions

In the description that follows, a number of terms are utilized extensively. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

$\text{A}\beta$ peptide is also known in the art as $\text{A}\beta$, β protein, β -A4 and A4. In the present invention, the $\text{A}\beta$ peptide may be comprised of peptides $\text{A}\beta_{1-39}$, $\text{A}\beta_{1-40}$, $\text{A}\beta_{1-41}$, $\text{A}\beta_{1-42}$, and $\text{A}\beta_{1-43}$. The most preferred embodiment of the invention makes use of $\text{A}\beta_{1-40}$. However, any of the $\text{A}\beta$ peptides may be employed according to the present invention. The sequence of $\text{A}\beta$ peptide is found in C. Hilbich *et al.*, *J. Mol. Biol.* 228:460-473 (1992).

Amyloid as is commonly known in the art, and as is intended in the present specification, is a form of aggregated protein.

A β Amyloid is an aggregated A β peptide. It is found in the brains of patients afflicted with AD and DS and may accumulate following head injuries.

5 **Physiological solution** as used in the present specification means a solution which comprises compounds at physiological pH, about 7.4, which closely represents a bodily or biological fluid, such as CSF, blood, plasma, et cetera.

10 **Zinc**, unless otherwise indicated, means salts of zinc, i.e., Zn²⁺ in any form, soluble or insoluble.

Copper(II), unless otherwise indicated, means salts of Cu(II), i.e., Cu²⁺ in any form, soluble or insoluble.

Copper(I), unless otherwise indicated, means salts of Cu(I), i.e., Cu⁺ in any form, soluble or insoluble.

15 **Biological fluid** means fluid obtained from a person or animal which is produced by said person or animal. Examples of biological fluids include but are not limited to cerebrospinal fluid (CSF), blood, serum, and plasma. In the present invention, biological fluid includes whole or any fraction of such fluids derived by purification by any means, *e.g.*, by ultrafiltration or chromatography.

20 The aim of the present invention is to clarify both the factors which contribute to the neurotoxicity of A β polymers and the mechanism which underlies their formation. These findings can then be used to (i) identify agents that can be used to decrease the neurotoxicity of A β , as well as the formation of A β polymers, and (ii) utilize such agents to develop methods of preventing,
25 treating or alleviating the symptoms of AD.

 The present invention relates to the unexpected discovery that A β peptides directly produce oxidative stress through the generation of abundant reactive oxygen species (ROS), which include hydroxyl radical (OH \cdot) and hydrogen peroxide (H₂O₂). The production of ROS occurs by a metal (Cu, Fe) dependant,

pH mediated mechanism, wherein the reduction of Cu(II) to Cu(I), or Fe(III) to Fe(II), is catalyzed by A β . A β is highly efficient at reducing Cu(II) and Fe(III).

All the redox properties of A β_{1-40} (the most abundant form of soluble A β) are exaggerated in A β_{1-42} . Additionally, A β_{1-42} , but not A β_{1-40} , recruits O₂ into spontaneous generation of another ROS, O₂⁻, which also occurs in a metal-dependent manner. The exaggerated redox activity of A β_{1-42} and its enhanced ability to generate ROS are likely to be the explanation for its neurotoxic properties. Interestingly, the rat homologue of A β , which has 3 substitutions that have been shown to attenuate zinc binding and zinc-mediated precipitation. The rat homologue also exhibits less redox activity than its human counterpart. This may explain why the rat is exceptional in that it is the only mammal that does not exhibit amyloid pathology with age. All other mammals analyzed to date possess the human A β sequence.

The sequence of ROS generation by A β follows the pathway of superoxide-dismutation, which leads to hydrogen peroxide production in a Cu/Fe-dependent manner. After forming H₂O₂, the hydroxyl radical (OH \cdot) is rapidly formed by a Fenton reaction with the Fe or Cu that is present, even when these metals are only at trace concentrations. The OH \cdot radical is very reactive and rapidly attacks the A β peptide, causing it to cross-link and polymerize. This is very likely to be the chemical mechanism that causes the covalent cross-linking that is seen in mature plaque amyloid. Importantly, the redox activity of A β is not attenuated by precipitation of the peptide, suggesting that, *in vivo*, amyloid deposits could be capable of generating ROS *in situ* on an enduring basis. This suggests that the major source of the oxidative stress in an AD-affected brain are amyloid deposits.

A model for free radical and amyloid formation in AD is shown in Fig. 12. The proposed mechanism is explained as follows.

(1) Soluble and precipitated A β species possess superoxide dismutase (SOD)-like activity. Superoxide (O₂⁻), the substrate for the dismutation, is generated both by spillover from mitochondrial respiratory metabolism, and by

A β ₁₋₄₂ itself (see FIG. 11). A β -mediated dismutation produces hydrogen peroxide (H₂O₂), requiring Cu(II) or Fe(III), which are reduced during the reaction. Since H⁺ is required for H₂O₂ production, an acidotic environment will increase the reaction.

(2) H₂O₂ is relatively stable, and freely permeable across cell membranes. Normally, it will be broken down by intercellular catalase or glutathione peroxidase.

(3) In aging and AD, levels of H₂O₂ are high, and catalase and peroxidase activities are low. If H₂O₂ is not completely catalyzed, it will react with reduced Cu(I) and Fe(II) in the vicinity of A β to generate the highly reduced Cu(I) and Fe(II) in the vicinity of A β to generate the highly reactive hydroxyl radical (OH•) by Fenton chemistry.

(4) OH• engenders a non-specific stress and inflammatory response in local tissue. Among the neurochemicals that are released from microglia and possibly neurons in the response are Zn(II), Cu(II) and soluble A β . Familial AD increases the likelihood that A β ₁₋₄₂ will be released at this point. Local acidosis is also part of the stress/inflammatory response. These factors combine to make A β precipitate and accumulate, presumably so that it may function *in situ* as an SOD, since these factors induce reversible polymerization. Hence, more soluble A β species decorate the perimeter of the accumulating plaque deposits.

(5) If A β encounters OH•, it will covalently cross-link during the oligomerization process, making it a more difficult accumulation to resolubilize, and leading to the formation of SDS-resistant oligomers characteristic of plaque amyloid.

(6) If A β ₁₋₄₂ accumulates, it has the property of recruiting O₂ as a substrate for the abundant production of O₂⁻ by a process that is still not understood. Since O₂ is abundant in the brain, A β ₁₋₄₂ is responsible for setting off a vicious cycle in which the accumulation of covalently linked A β is a product of the unusual ability of A β to reduce O₂, and feed an abundant substrate (O₂⁻) to itself for dismutation, leading to OH• formation. The production of abundant free

radicals by the accumulating amyloid may further damage many systems including metal regulatory proteins, thus compounding the problem. This suggests that the major source of the oxidative stress in an AD-affected brain are amyloid deposits.

5 The metal-dependent chemistry of A β -mediated superoxide dismutation is reminiscent of the activity of superoxide dismutase (SOD). Interestingly, mutations of SOD cause amyotrophic lateral sclerosis, another neurodegenerative disorder. SOD is predominantly intracellular, whereas A β is constitutively found in the extracellular spaces where it accumulates. Investigation of A β by laser
10 flash photolysis confirmed the peptide's SOD-like activity, suggesting that A β may be an anti-oxidant under physiological circumstances. Since H₂O₂ has been shown to induce the production of A β , the accumulation of A β in AD may reflect a response to an oxidant stress paradoxically caused by A β excess. This may cause and, in turn, be compounded by, damage to the biometal homeostatic mechanisms in the brain environment.
15

Thus, it has recently been discovered (i) that much of the A β aggregate in AD-affected brain is held together by zinc and copper, (ii) that A β peptides exhibit Fe/Cu-dependent redox activity similar to that of SOD, (iii) that A β ₁₋₄₂ is especially redox reactive and has the unusual property of reducing O₂ to O₂⁻, and
20 (iv) that deregulation of A β redox reactivity causes the peptide to conveniently polymerize. Since these reactions must be strongly implicated in the pathogenetic events of AD, they offer promising targets for therapeutic drug design.

The discovery that A β can generate H₂O₂ and Cu(I), both of which are associated with neurotoxic effects, offers an explanation for the neurotoxicity of
25 A β polymers. These findings suggest that it may be possible to lessen the neurotoxicity of A β by controlling factors which alter the concentrations of Cu(I) and ROS, including hydrogen peroxide, being generated by accumulated and soluble A β . It has been discovered that manipulation of factors such as zinc, copper, and pH can result in altered Cu(I) and H₂O₂ production by A β .
30 Therefore, agents identified as being useful for the adjustment of the pH and

levels of zinc and copper of the brain interstitium can be used to adjust the concentration of Cu(I) and H_2O_2 , and can therefore be used to reduce the neurotoxic burden. Such agents will thus be a means of treating Alzheimer's disease.

5 Thus, one object of the present invention is to provide a method for the identification of agents to be used in the treatment of AD. As may be understood by reference to the Examples below, agents to be used in the treatment of AD include:

- 10 (a) agents that reduce the amount of Cu(I) or Fe(II) produced by $A\beta$;
 (b) agents that promote or inhibit the production of hydrogen peroxide by $A\beta$;
 (c) agents that inhibit the production of O_2^- by $A\beta$;
 (d) agents that inhibit the production of OH^\bullet .

15 Of course, as aggregation and especially crosslinking of $A\beta$ contributes to the neurotoxic burden, agents which have been identified to have the activities listed above may then also be subjected to tests which determine if an agent is capable of inhibiting oligomerization by $A\beta$ (see Example 1).

 Agents identified as having the above-listed activities may then be tested for their ability to reduce the neurotoxicity of both soluble and crosslinked $A\beta$.

20 Thus, in one aspect, the invention relates to a method for the identification of an agent to be used in the treatment of AD, wherein the agent is capable of altering, and preferably decreasing, the production of Cu(I) by $A\beta$, the method comprising:

- 25 (a) adding Cu(II) to a first $A\beta$ sample;
 (b) allowing the first sample to incubate for an amount of time sufficient to allow said first sample to generate Cu(I);
 (c) adding Cu(II) to a second $A\beta$ sample, the second sample additionally comprising a candidate pharmacological agent;
 (d) allowing the second sample to incubate for the same
30 amount of time as the first sample;

(e) determining the amount of Cu(I) produced by the first sample and the second sample; and

(f) comparing the amount of Cu(I) produced by the first sample to the amount of Cu(I) produced by the second sample;

5 whereby a difference in the amount of Cu(I) produced by the first sample as compared to the second sample indicates that the candidate pharmacological agent has altered the production of Cu(I) by A β . Of course, where the amount of Cu(I) is lower in the second sample than in the first sample, this will indicate that the agent has decreased Cu(I) production.

10 In a preferred embodiment, the amount of Cu(I) present in said first and said second sample is determined by

(a) adding a complexing agent to said first and said second sample, wherein said complexing agent is capable of combining with Cu(I) to form a complex compound, wherein said complex compound has an optimal
15 visible absorption wavelength;

(b) measuring the absorbancy of said first and said second sample; and

(c) calculating the concentration of Cu(I) in said first and said second sample using the absorbancy obtained in step (b).

20 In a more preferred embodiment, the complexing agent is bathocuproinedisulfonic (BC) anion. The concentration of Cu⁺ produced by A β may then be calculated on the basis of the absorbance of the sample at about 478 nm to about 488 nm, more preferable about 480 to about 486 nm, and most preferably about 483 nm.

25 In an even more preferred embodiment, the above-described method may be performed in a microtiter plate, and the absorbancy measurement is performed by a plate reader, thus allowing large numbers of candidate pharmacological compounds to be tested simultaneously.

30 In another aspect, the invention relates to a method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of

altering, and preferably decreasing, the production of Fe(II) by A β , said method comprising:

- (a) adding Fe(III) to a first A β sample;
- (b) allowing said first sample to incubate for an amount of time sufficient to allow said first sample to generate Cu⁺;
- (c) adding Fe(III) to a second A β sample, said second sample additionally comprising a candidate pharmacological agent;
- (d) allowing said second sample to incubate for the same amount of time as said first sample;
- (e) determining the amount of Fe(II) produced by said first sample and said second sample; and
- (f) comparing the amount of Fe(II) present in said first sample to the amount of Fe(II) present in said second sample;

whereby a difference in the amount of Fe(II) present in said first sample as compared to said second sample indicates that said candidate pharmacological agent has altered the production of Fe(II) by A β . Of course, where the amount of Fe(II) is lower in the second sample than in the first sample, this will indicate that the agent has decreased Fe(II) production.

In a preferred embodiment, the amount of Fe(II) present is determined by using a spectrophotometric method analogous to that used for the determination of Cu(I), above. In this method, the complexing agent is batho-phenanthrolinedisulfonic (BP) anion. The concentration of Fe²⁺-BP produced by A β may then be calculated on the basis of the absorbance of the sample at about 530 to about 540 nm, more preferably about 533 nm to about 538 nm, and most preferably about 535 nm.

In an even more preferred embodiment, the above-described method may be preformed in a microtiter plate, and the absorbancy measurement is performed by a plate reader, thus allowing large numbers of candidate pharmacological compounds to be tested simultaneously.

In yet another aspect, the invention relates to a method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of altering the production of H_2O_2 by $A\beta$, said method comprising:

- (a) adding Cu(II) or Fe(III) to a first $A\beta$ sample;
- 5 (b) allowing said first sample to incubate for an amount of time sufficient to allow said first sample to generate H_2O_2 ;
- (c) adding Cu(II) or Fe(III) to a second $A\beta$ sample, said second sample additionally comprising a candidate pharmacological agent;
- (d) allowing said second sample to incubate for the same
10 amount of time as said first sample;
- (e) determining the amount of H_2O_2 produced by said first sample and said second sample; and
- (f) comparing the amount of H_2O_2 present in said first sample to the amount of H_2O_2 present in said second sample;

15 whereby a difference in the amount of H_2O_2 present in said first sample as compared to said second sample indicates that said candidate pharmacological agent has altered the production of H_2O_2 by $A\beta$. As will be understood by one of ordinary skill in the art, this method may be used to detect agents which decrease the amount of H_2O_2 produced (in which case the amount of H_2O_2 will
20 be lower in the second sample than in the first sample), or to increase the amount of H_2O_2 produced (in which case the amount of H_2O_2 will be lower in the first sample than in the second sample).

In a preferred embodiment, the determination of the amount of H_2O_2 present in said first and said second sample is determined by

- 25 (a) adding catalase to a first aliquot of said first sample obtained in step (a) of claim 1 in an amount sufficient to break down all of the H_2O_2 generated by said sample;
- (b) adding TCEP, in an amount sufficient to capture all of the H_2O_2 generated by said samples, to
30 (i) said first aliquot

(ii) a second aliquot of said first sample obtained in step (a) of claim 1; and

(iii) said second sample obtained in step (b) of claim 1;

(c) incubating the samples obtained in step (b) for an amount of time sufficient to allow the TCEP to capture all of the H_2O_2 ;

(d) adding DTNB to said samples obtained in step (c);

(e) incubating said samples obtained in step (d) for an amount of time sufficient to generate TMB;

(f) measuring the absorbancy at about 407 to about 417 nm of said samples obtained in step (e); and

(g) calculating the concentration of H_2O_2 in said first and said second sample using the absorbancies obtained in step (f). In a preferred embodiment, the absorbancy of TMB is measured at about 412 nm.

In a preferred embodiment, the above-described method is performed in a microtiter plate, and the absorbancy measurement is performed by a plate reader, thus making it possible to screen large numbers of candidate pharmacological agent simultaneously.

In another embodiment, the invention provides a method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of decreasing the production of O_2^- by $A\beta$, said method comprising:

(a) adding $A\beta$ and to a first buffer sample having an O_2 tension greater than 0;

(b) allowing said first sample to incubate for an amount of time sufficient to allow said first sample to generate O_2^- ;

(c) adding $A\beta$ and a candidate pharmacological agent to a second buffer sample having an O_2 tension greater than 0;;

(d) allowing said second sample to incubate for the same amount of time as said first sample;

(e) determining the amount of O_2^- produced by said first sample and said second sample; and

(f) comparing the amount of O_2^- present in said first sample to the amount of O_2^- present in said second sample; whereby a difference in the amount of O_2^- present in said first sample as compared to said second sample indicates that said candidate pharmacological agent has altered the production of O_2^- by $A\beta$. In a preferred embodiment, the $A\beta$ used is $A\beta_{1-42}$.

Of course, the amount of O_2^- produced by $A\beta$ may be measured by any method known to those of ordinary skill in the art. In a preferred embodiment, the determination of the amount of O_2^- present in said samples is accomplished by measuring the absorbancy of the sample at about 250 nm.

Because the ability of $A\beta$ to generate H_2O_2 from O_2^- may in many instances be beneficial, in a preferred embodiment, the invention also relates to a method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of interfering with the interaction of O_2^- and $A\beta$ to produce O_2^- , without interfering with the SOD-like activity of $A\beta$, said method comprising:

(a) identifying an agent capable of decreasing the production of O_2^- by $A\beta$; and

(b) determining the ability of said agent to alter the SOD-like activity of $A\beta$. In a preferred embodiment, the determination of the ability of said agent to alter the SOD-like activity of $A\beta$ is made by determining whether $A\beta$ is capable of catalytically producing Cu(I), Fe(II) or H_2O_2 . Methods, besides those which are disclosed elsewhere in this application, for determining if $A\beta$ is capable of catalytically producing Cu(I), Fe(II) or H_2O_2 are well known to those of ordinary skill in the art. In particular, the catalytic production of H_2O_2 may be determined by using laser flash photolysis or pulse radiolysis (G. Peters and M.A. J. Rodgers, *Biochim. Biophys. Acta* 637: 43-52 (1981)).

In another aspect, candidate pharmacological agents which have been identified by one or more of the above screening assays can undergo further screening to determine if the agents are capable of altering, and preferably

reducing or eliminating, A β -mediated toxicity in cell culture. Such assays include the MTT assay, which measures the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyl tetrazolium bromide (MTT) to a colored formazon (Hansen *et al.*, 1989). Although alternatives have not been ruled out (see Burdon *et al.*, 1993), the major site of MTT reduction is thought to be at two stages of electron transport, the cytochrome oxidase and ubiquinone of mitochondria (Slater *et al.*, 1963). A second cytotoxic assay is the release of lactic dehydrogenase (LDH) from cells, a measurement routinely used to quantitate cytotoxicity in cultured CNS cells (Choi, 1987). While MTT measures primarily early redox changes within the cell reflecting the integrity of the electron transport chain, the release of LDH is thought to be through cell lysis. A third assay is visual counting in conjunction with trypan blue exclusion. Other commercially available assays for neurotoxicity, including the Live-Dead assay, may also be used to determine if a candidate compound which alters Cu(I), Fe(II), H₂O₂, OH \cdot , and O₂ \cdot production, or alters copper-induced, pH dependent aggregation and crosslinking of A β , is also capable of reducing the neurotoxicity of A β .

Thus, in another preferred embodiment, the invention relates to a method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of reducing the toxicity of A β , said method comprising:

- (a) adding A β to a first cell culture;
- (b) adding A β to a second cell culture, said second cell culture additionally containing a candidate pharmacological agent;
- (c) determining the level of neurotoxicity of A β in said first and said second samples; and
- (d) comparing the level of neurotoxicity of A β in said first and said second samples,

whereby a lower neurotoxicity level in said second sample as compared to said first sample indicates that said candidate pharmacological agent has reduced the neurotoxicity of A β , and is thereby capable of being used to treat AD.

Assays which can be used to determine the neurotoxicity of a candidate agent include, but are not limited to, the MTT assay and the LDH release assay, as described in Behl *et al.* (*Cell* 77: 817-827 (1994)) and the Live/Dead EukoLight Viability/Cytotoxicity Assay, commercially available from Molecular Probes, Inc. (Eugene, OR).

Cells types which may be used for these neurotoxicity assays include both cancer cells and primary cells, such as rat primary frontal neuronal cells.

Candidate pharmacological agents to be tested in any of the above-described methods will be broad-ranging but can be classified as follows:

Candidate pharmacological agents for the alteration of the SOD-like activity of A β will be broad-ranging but can be classified as follows:

Agents which modify the availability of zinc or copper for interaction with A β : They include chelating agents such as desferrioxamine, but also

include amino acids histidine and cysteine which bind free zinc, and are thought to be involved in bringing zinc from the plasma across the blood-brain barrier (BBB). These agents include all classes of specific zinc chelating agents, and combinations of non-specific chelating agents capable of chelating zinc such as EDTA (Edetic acid, N,N'-1,2-Ethane diylbis[N-(carboxymethyl)glycine] or (ethylenedinitrilo)tetraacetic acid, entry 3490 in Merck Index 10th edition) and all salts of EDTA, and/or phytic acid [myo-Inositol hexakis(dihydrogen phosphate), entry 7269 in the Merck Index 10th edition] and phytate salts. Preferred candidate agents within this class include bathocuproine and bathophenanthroline

Miscellaneous: Because there is no precedent for an effective anti-amyloidotic pharmaceutical, it is reasonable to serendipitously try out compounds which may have access to the brain compartment for their ability to inhibit either Cu⁺ or H₂O₂ production by A β . These compounds include dye compounds, heparin, heparan sulfate, and anti-oxidants, *e.g.*, ascorbate, trolox and tocopherols.

In the present invention, the A β used may be any form of A β . In a preferred embodiment, the A β used is selected from the group consisting of A β ₁₋₃₉, A β ₁₋₄₀, A β ₁₋₄₁, A β ₁₋₄₂, and A β ₁₋₄₃. Even more preferably, the A β used is A β ₁₋₄₀ or A β ₁₋₄₂. The most preferred embodiment of the invention makes use of A β ₁₋₄₀. The sequence of A β peptide is found in C. Hilbich *et al.*, *J. Mol. Biol.* 228:460-473 (1992).

The pH of the various reaction mixtures are preferably close to neutral (about 7.4). The pH, therefore, may range from about 6.6 to about 8, preferably from about 6.6 to about 7.8, and most preferably about 7.4.

Buffers which can be used in the methods of the present invention include, but are not limited to, PBS, Tris-chloride and Tris-base, MOPS, HEPES, bicarbonate, Krebs, and Tyrode's. The concentration of the buffers may be between about 10 mM and about 500 mM. Because of the nature of the assays which are included in the methods of the claimed invention, when choosing a buffer, it must be borne in mind that spontaneous free radical production within a given buffer might interfere with the reactions. For this reason, PBS is the preferred buffer for use in the methods of the invention, although other buffers may be used provided that proper controls are used to correct for the above-mentioned free radical formation of a given buffer.

Cu(II) must be present in the reaction mixture for A β to produce Cu⁺. Any salt of Cu(II) may be used to satisfy this requirement, including, but not limited to, CuCl₂, Cu(NO₃)₂, *etc.* Concentrations of copper from at least about 1 μ M may be used; most preferable, a copper concentration of about 10 μ M is to be included in the reaction mixture.

Similarly, a redox active metal such as Cu(II) or Fe(III) must be present in the reaction mixture for A β to catalytically produce H₂O₂. Any salt of Cu(II) may be used to satisfy this requirement, including, but not limited to, CuCl₂, Cu(NO₃)₂, *etc.* Similarly, and salt of Fe(III) may be used in accordance with the invention, such as FeCl₃. Concentrations of copper or iron from at least about 1

μM may be used; most preferably, a copper or iron concentration of about $10\ \mu\text{M}$ is to be included in the reaction mixture.

The present invention may be practiced at temperatures ranging from about 25°C to about 40°C . The preferred temperature range is from about 30°C to about 40°C . The most preferred temperature for the practice of the present invention is about 37°C , *i.e.*, human body temperature.

The production of Cu^{+} and H_2O_2 by $\text{A}\beta$ peptide occurs at near-instantaneous rate. Hence, the measurement of the concentration of Cu^{+} or H_2O_2 produced may be performed by the present methods substantially immediately after the addition of Cu(II) to the $\text{A}\beta$ peptide. However, if desired, the reaction may be allowed to proceed longer. In a preferred embodiment of the invention, the reaction is carried out for about 30 minutes.

The invention may also be carried out in the presence of biological fluids, such as the preferred biological fluid, CSF, to closely simulate actual physiological conditions. Of course, such fluids will already contain $\text{A}\beta$, so that where the methods of the invention are to be carried out utilizing a biological fluid such as CSF, no further $\text{A}\beta$ peptide will be added to the sample. The biological fluid may be used directly or diluted from about 1:1,000 to about 1:5 fold.

The amount of H_2O_2 , Cu(I) or Fe(II) produced by a sample may be measured by any standard assay for H_2O_2 , Cu(I) or Fe(II) . For example, the PeroXOquant Quantitative Peroxide Assay (Pierce, Rockford, IL) may be used to determine the amount of H_2O_2 produced. Fe(II) may be determined using the spectrophotometric method of Linert *et al.* (*Biochim. Biophys. Acta* 1316:160-168 (1996)). Other such methods will be readily apparent to those of ordinary skill in the art.

In a preferred embodiment, the H_2O_2 or Cu^{+} produced by the sample is complexed with a complexing agent having an optimal visible absorption wavelength. The amount of H_2O_2 or Cu^{+} produced by a sample is then detected using optical spectrophotometry (see Example 2).

In a preferred embodiment, the complexing agent to be used for the determination of the amount of Cu^+ produced is bathocuproinedisulfonic anion (BC), (see Example 2); the complex Cu^+ -BC has an optimal visible absorption wavelength of about 483 nm. As is mentioned above, $\text{A}\beta$ will produce H_2O_2 and Cu^+ almost immediately following the addition of Cu(II) and Zn(II) to the reaction mixture. Thus, BC may be added to the reaction immediately following the addition of Cu(II) and Zn(II) to the $\text{A}\beta$ samples. The concentration of BC to be achieved in a sample is between about 10 μM to about 400 μM , more preferably about 75 μM to about 300 μM , and still more preferably about 150 μM to about 275 μM . In the most preferred embodiment, the concentration of BC to be achieved in a sample is about 200 μM . Of course, one of ordinary skill in the art can easily optimize the concentration of BC to be added with no more than routine experimentation.

Where the amount of Fe(II) produced is to be determined, the complexing agent to be used for the determination of the amount of Fe(II) produced is bathophenanthrolinedisulfonic (BP) anion, (see Example 2); the complex Fe^{2+} -BP has an optimal visible absorption wavelength of about 535 nm. As is mentioned above, $\text{A}\beta$ will produce H_2O_2 and Fe(II) almost immediately following the addition of Fe(III) and Zn(II) to the reaction mixture. Thus, BP may be added to the reaction immediately following the addition of Fe(III) and Zn(II) to the $\text{A}\beta$ samples. The concentration of BP to be achieved in a sample is between about 10 μM to about 400 μM , more preferably about 75 μM to about 300 μM , and still more preferably about 150 μM to about 275 μM . In the most preferred embodiment, the concentration of BP to be achieved in a sample is about 200 μM . Of course, one of ordinary skill in the art can easily optimize the concentration of BP to be added with no more than routine experimentation.

The above-described spectrophotometric assays may be used to determine the concentration of Cu^+ or Fe^{2+} , as is described in Example 2.

Each of the assays of the present invention is ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being

compartmentalized to receive in close confinement therein one or more container means, such as vials, tubes, and the like, each of said container means comprising one of the separate elements of the assay to be used in the method. For example, there may be provided a container means containing standard solutions of the A β peptide or lyophilized A β peptide and a container means containing a standard solution or varying amounts of a salt of redox active metal, such as Cu(II) or Fe(III), in any form, *i.e.*, in solution or dried, soluble or insoluble, in addition to further carrier means containing varying amounts and/or concentrations of reagents used in the present methods. For example, solutions to be used for the determination of Cu(I) or Fe(II) as described in Example 2 will include BC anion and BP anion, respectively. Similarly, solutions to be used for the determination of H₂O₂ as described in Example 2 include TCEP and DTNB, as well as catalase (10U/ml). Standard solutions of A β peptide preferably have concentrations above about 10 μ M, more preferably from about 10 to about 25 μ M or if the peptide is provided in its lyophilized form, it is provided in an amount which can be solubilized to said concentrations by adding an aqueous buffer or physiological solution. The standard solutions of analytes may be used to prepare control and test reaction mixtures for comparison, according to the methods of the present invention.

Examples

Example 1

Copper-Induced, pH Dependent Aggregation of A β

Materials and Methods

a) Preparation of A β Stock

Human A β_{1-40} peptide was synthesized, purified and characterized by HPLC analysis, amino acid analysis and mass spectroscopy by W.M. Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT). Synthetic A β peptide solutions were dissolved in trifluoroethanol (30 % in Milli-Q water (Millipore Corporation, Milford, MA)) or 20 mM Hepes (pH 8.5) at a concentration of 0.5-1.0 g/ml, centrifuged for 20 min. at 10 000g and the supernatant (stock A β_{1-40}) used for subsequent aggregation assays on the day of the experiment. The concentration of stock A β_{1-40} was determined by UV spectroscopy at 214 nm or by Micro BCA protein assay (Pierce, Rockford, IL). The Micro BCA assay was performed by adding 10 μ l of stock A β_{1-40} (or bovine serum albumin standard) to 140 μ l of distilled water, and then adding an equal volume of supernatant (150 μ l) to a 96-well plate and measuring the absorbance at 562 nm. The concentration of A β_{1-40} was determined from the BSA standard curve. Prior to use all buffers and stock solutions of metal ions were filtered though a 0.22 μ m filter (Gelan Sciences, Ann Arbor, MI) to remove any particulate matter. All metal ions were the chloride salt, except lead nitrate.

b) Aggregation Assays

A β_{1-40} stock was diluted to 2.5 μ M in 150 mM NaCl and 20 mM glycine (pH 3-4.5), mes (pH 5-6.2) or Hepes (pH 6.4-8.8), with or without metal ions,

incubated (30 min., 37 °C), centrifuged (20 min., 10 000g). The amount of protein in the supernatant was determined by the Micro BCA protein assay as described above.

c) Turbidometric Assays

5 Turbidity measurements were performed as described by Huang *et al.*,
Journal of Biological Chemistry (submitted), except A β ₁₋₄₀ stock was brought to
10 $10\ \mu\text{M}$ (300 μl) in 20 mM HEPES buffer, 150 mM NaCl (pH 6.6, 6.8 or 7.4) with
or without metal ions prior to incubation (30 min., 37 °C). To investigate the pH
reversibility of Cu²⁺-induced A β aggregation, 25 μM A β ₁₋₄₀ and 25 μM Cu²⁺ were
15 mixed in 67 mM phosphate buffer, 150 mM NaCl (pH 7.4) and turbidity
measurements were taken at four 1 min. intervals. Subsequently, 20 μl aliquots
of 10 mM EDTA or 10 mM Cu²⁺ were added into the wells alternatively, and,
following a 2 min. delay, a further four readings were taken at 1 min. intervals.
After the final EDTA addition and turbidity reading, the mixtures were incubated
20 for an additional 30 min. before taking final readings. To investigate the
reversibility of pH mediated Cu²⁺-induced A β ₁₋₄₀ aggregation, 10 μM A β ₁₋₄₀ and
30 μM Cu²⁺ were mixed in 67 mM phosphate buffer, 150 mM NaCl (pH 7.4) and
an initial turbidity measurement taken. Subsequently, the pH of the solution was
successively decreased to 6.6 and then increased back to 7.5. The pH of the
25 reaction was monitored with a microprobe (Lazar Research Laboratories Inc., Los
Angeles, CA) and the turbidity read at 5 min. intervals for up to 30 min. This
cycle was repeated three times.

d) Immunofiltration Detection of Low Concentrations of A β ₁₋₄₀ Aggregate

Physiological concentrations of A β ₁₋₄₀ (8 nM) were brought to 150 mM
25 NaCl, 20 mM HEPES (pH 6.6 or 7.4), 100 nM BSA with CuCl₂ (0, 0.1, 0.2, 0.5
and 2 μM) and incubated (30 min., 37°C). The reaction mixtures (200 μl) were

then placed into the 96-well Easy-Titer ELISA system (Pierce, Rockford, IL) and filtered through a 0.22 μ m cellulose acetate filter (MSI, Westboro, MA). Aggregated particles were fixed to the membrane (0.1% glutaraldehyde, 15 min.), washed thoroughly and then probed with the anti-A β mAb 6E10 (Senetek, Maryland Heights, MI). Blots were washed and exposed to film in the presence of ECL chemiluminescence reagents (Amersham, Buckinghamshire, England). Immunoreactivity was quantified by transmittance analysis of ECL film from the immunoblots.

e) A β metal-capture ELISA

A β_{1-40} (1.5 ng/well) was incubated (37°C, 2 hr) in the wells of Cu²⁺ coated microtiter plates (Xenopore, Hawthorne, NJ) with increasing concentrations of Cu²⁺ (1-100 nM) as described by Moir *et al.*, *Journal of Biological Chemistry* (submitted). Remaining ligand binding sites on well surfaces were blocked with 2% gelatin in tris-buffered saline (TBS) (3 hr at 37°C) prior to overnight incubation at room temperature with the anti-A β mAb 6E10 (Senetek, Maryland Heights, MI). Anti-mouse IgG coupled to horseradish peroxidase was then added to each well and incubated for 3 hr at 37°C. Bound antibodies were detected by a 30 minute incubation with stable peroxidase substrate buffer/3,3',5,5'-Tetramethyl benzidine (SPSB/TMB) buffer, followed by the addition of 2 M sulfuric acid and measurement of the increase in absorbance at 450 nm.

f) Extraction of A β from post-mortem brain tissue

Identical regions of frontal cortex (0.5g) from post-mortem brains of individuals with AD, as well as non-AD conditions, were homogenized in TBS, pH 4.7 \pm metal chelators. The homogenate was centrifuged and samples of the soluble supernatant as well as the pellet were extracted into SDS sample buffer and assayed for A β content by western blotting using monoclonal antibody

(mAb) WO2. The data shows a typical (of n=12 comparisons) result comparing the amount of A β extracted into the supernatant phase in AD compared to control (young adult) samples. N,N,N',N'-tetrakis [2-pyridyl-methyl] ethylenediamine (TPEN) (5 μ M) allows the visualization of a population of pelletable A β that had not previously been recognized in unaffected brain samples (Fig. 8).

g) A β cross-linking by copper

Cu²⁺-induced SDS-resistant oligomerization of A β : A β ₁₋₄₀ (2.5 μ M), 150 mM NaCl, 20 mM hepes (pH 6.6, 7.4, 9) with or without ZnCl₂ or CuCl₂. Following incubation (37°C), aliquots of each reaction (2 ng peptide) were collected at 0 d, 1 d, 3 d and 5 d and western blotted using anti-A β monoclonal antibody 8E10. Migration of the molecular size markers are indicated (kDa). The dimer formed under these conditions has been found to be covalently linked. Cu(II) (2-30 μ M) induced and covalent oligomerization of peptide. Co-incubation with similar concentrations of Zn(II) accelerates the bridging, but zinc alone has no effect. The antioxidant sodium metabisulphite moderately attenuates the reaction, while ascorbic acid dramatically accelerates A β bridging. This suggests reduction of Cu(II) to Cu(I) with the latter mediating covalent bridging of A β . Mannitol also abolishes the cross-linking, suggesting that the bridging is mediated by the generation of the hydroxyl radical by a Fenton reaction that recruits Cu(I) (Fig. 9). It should be noted that other means of visualizing and/or determining the presence or absence of crosslinking other than western blot analysis may be used. Such other means include but are not limited to density sedimentation by centrifugation of the samples.

Results

It has previously been reported that Zn^{2+} induces rapid precipitation of A β *in vitro* (Bush, A.I., *et al.*, *Science* 268:1921 (1995)). This metal has an abnormal metabolism in AD and is highly concentrated in brain regions where A β precipitates. The present data indicate that under very slightly acidic conditions, such as in the lactic acidotic AD brain, Cu^{2+} strikingly induces the precipitation of A β through an unknown conformational shift. pH alone dramatically affects A β solubility, inducing precipitation when the pH of the incubation approaches the pl of the peptide (pH 5-6). Zinc induces 40-50% of the peptide to precipitate at pH > 6.2, below pH 6.2 the precipitating effects of Zn^{2+} and acid are not summative. At pH \leq 5, Zn^{2+} has little effect upon A β solubility. Cu^{2+} is more effective than Zn^{2+} in precipitating A β and even induces precipitation at the physiologically relevant pH 6-7. Copper-induced precipitation of A β occurs as the pH falls below 7.0, comparable with conditions of acidosis (Yates, C.M., *et al.*, *J. Neurochem.* 55:1624 (1990)) in the AD brain. Investigation of the precipitating effects of a host or other metal ions in this system indicated that metal ion precipitation of A β was limited to copper and zinc, as illustrated, although Fe(II) possesses a partial capacity to induce precipitation (Bush, A.I., *et al.*, *Science* 268:1921 (1995)).

On the basis these *in vitro* findings, the possibility that A β deposits in the Ad-affected brain may be held in assembly by zinc and copper ions was investigated. Roher and colleagues have recently shown that much of the A β that deposits in Ad-affected cortex can be solubilized in water (Roher, A.E., *et al.*, *J. Biol. Chem.* 271:20631 (1996)). Supporting the clinical relevance of *in vitro* findings, it has recently been demonstrated that metal chelators increase the amount of A β extracted by Roher's technique (in neutral saline buffer), and that the extraction of A β is increased as the chelator employed has a higher affinity for zinc or copper (FIG. 8). Hence TPEN is highly efficient in extracting A β , as are TETA, and bathocuproene, EGTA and EDTA are less efficient, requiring

higher concentrations 91 mM) to achieve the same level of recovery as say, TPEN (5 μ M). Zinc and copper ions (5-50 μ M) added back to the extracting solution abolish the recovery of A μ (which is subsequently extracted by the SDS sample buffer in the pellet fraction of the centrifuged brain homogenate suspension), but Ca²⁺ and Mg²⁺ added back to the chelator-mediated extracts of A β cannot abolish A β resolubilization from Ad-affected tissue even when these metal ions are present in millimolar concentrations.

Importantly, atomic absorption spectrophotometry assays of the metal content of the chelator-mediated extracts confirms that Cu and Zn are co-released with A β by the chelators, along with lower concentrations of Fe. These data strongly indicate that A β deposits (probably of the amorphous type) are held together by Cu and Zn and may also contain Fe. Interestingly, A β is not extractable from control brain without the use of chelators. This suggests that metal-assembled A β deposits may be the earliest step in the evolution of A β plaque pathology.

These findings propelled further inquiries into chemistry of metal ion- A β interaction. The precipitating effects upon A β of Zn(II) and Cu(II) were found to be qualitatively different. Zn-mediated aggregation is reversible with chelation and is not associated with neurotoxicity in primary neuronal cell cultures, whereas Cu-mediated aggregation is accompanied by the slow formation of covalently-bonded SDS-resistant dimers and induction of neurotoxicity. These neurotoxic SDS-resistant dimers are similar to those described by Roher (Roher, A.E., *et al.*, *J. Biol. Chem.* 271:20631 (1996)).

To accurately quantitate the effects of different metals and pH on A β solubility, synthetic human A β ₁₋₄₀ (2.5 μ M) was incubated (37°C) in the presence of metal ions at various pH for 30 min. The resultant aggregated particles were sedimented by centrifugation to permit determination of soluble A β ₁₋₄₀ in the supernatant. To determine the centrifugation time required to completely sediment the aggregated particles generated under these conditions, A β ₁₋₄₀ was incubated for 30 min at 37°C with no metal, Zn²⁺ (100 μ M), Cu²⁺ (100 μ M) and

pH (5.5). Reaction mixtures were centrifuged at 10 000g for different times, or ultracentrifuged at 100 000g for 1 h. (FIG. 1). Figure 1 shows the proportion of soluble A β ₁₋₄₀ remaining following centrifugation of reaction mixtures. A β ₁₋₄₀ was incubated (30 min., 37 °C) with no metal, under acidic conditions (pH 5.5), Zn²⁺ (100 μ M) or Cu²⁺ (100 μ M), and centrifuged at 10 000g for different time intervals, or at 100,000g (ultracentrifuged) for 1 h for comparison. All data points are means \pm SD, n = 3.

Given that conformational changes within the N-terminal domain of A β are induced by modulating [H⁺] (Soto, C., *et al.*, *J. Neurochem.* 63:1191-1198 (1994)), and that there is a metal (Zn²⁺) binding domain in the same region, experiments were designed to determine whether there was a synergistic effect of pH on metal ion-induced A β aggregation. A β ₁₋₄₀ was incubated with different bioessential metal ions at pH 6.6, 6.8 and 7.4. The results are shown in FIG. 2A, where "all metals" indicates incubation with a combination containing each metal ion at the nominated concentrations, concurrently. FIG. 2A shows the proportion of soluble A β ₁₋₄₀ remaining in the supernatant after incubation (30 min., 37°C) with various metal ions at pH 6.6, 6.8 or 7.4 after centrifugation (10,000g, 20 min.),

The [H⁺] chosen represented the most extreme, yet physiologically plausible [H⁺] that A β ₁₋₄₀ would be likely to encounter *in vivo*. The ability of different bioessential metal ions to aggregate A β ₁₋₄₀ at increasing H⁺ concentrations fell into two groups; Mg²⁺, Ca²⁺, Al³⁺, Co²⁺, Hg²⁺, Fe³⁺, Pb²⁺ and Cu²⁺ showed increasing sensitivity to induce A β ₁₋₄₀ aggregation, while Fe²⁺, Mn²⁺, Ni²⁺, and Zn²⁺ were insensitive to alterations in [H⁺] in their ability to aggregate A β ₁₋₄₀. Cu²⁺ and Hg²⁺ induced most aggregation as the [H⁺] increased, although the [H⁺] insensitive Zn²⁺-induced aggregation produced a similar amount of aggregation. Fe²⁺, but not Fe³⁺, also induced considerable aggregation as the [H⁺] increased, possibly reflecting increased aggregation as a result of increased crosslinking of the peptide.

Similar results were obtained when these experiments were repeated using turbidometry as an index of aggregation (FIG. 2B). The data indicate the absorbance changes between reaction mixtures with and without metal ions at pH 6.6, 6.8 or 7.4. Thus, A β ₁₋₄₀ has both a pH insensitive and a pH sensitive metal binding site. At higher concentrations of metal ions this pattern was repeated, except Co²⁺ and Al³⁺-induced A β aggregation became pH insensitive, and Mn became sensitive (FIG. 2C).

Since ⁶⁴Cu is impractically short-lived (t_{1/2} = 13 h), a novel metal-capture ELISA assay was used to perform competition analysis of A β ₁₋₄₀ binding to a microtiter plate impregnated with Cu²⁺, as described in Materials and Methods. Results are shown in FIG. 3. All assays were performed in triplicate and are means \pm SD, n=3. Competition analysis revealed that A β ₁₋₄₀ has at least one high affinity, saturable Cu²⁺ binding site with a K_d = 900 pM at pH 7.4 (FIG. 3). The affinity of A β for Cu²⁺ is higher than that for Zn²⁺ (Bush, A.I., *et al.*, *J. Biol. Chem.* 269:12152 (1994)). Since Cu²⁺ does not decrease Zn²⁺-induced aggregation (Bush, A.I., *et al.*, *J. Biol. Chem.* 269:12152 (1994)), indicating Cu²⁺ does not displace bound Zn²⁺, there are likely to be two separate metal binding sites. This is supported by the fact that there is both a pH sensitive and insensitive interaction with different metal ions.

Since the conformational state and solubility of A β is altered at different pH (Soto, C., *et al.*, *J. Neurochem.* 63:1191-1198 (1994)), the effects of [H⁺] on Zn²⁺- and Cu²⁺-induced A β ₁₋₄₀ aggregation were studied. Results are shown in FIGS. 4A, 4B and 4C. FIG. 4A shows the proportion of soluble A β ₁₋₄₀ remaining in the supernatant following incubation (30 min., 37°C) at pH 3.0-8.8 in buffered saline \pm Zn²⁺ (30 μ M) or Cu²⁺ (30 μ M) and centrifugation (10 000g, 20 min.), expressed as a percentage of starting peptide. All data points are means \pm SD, n=3. [H⁺] alone precipitates A β ₁₋₄₀ (2.5 μ M) as the solution is lowered below pH 7.4, and dramatically once the pH falls below 6.3 (FIG. 4A). At pH 5.0, 80% of the peptide is precipitated, but the peptide is not aggregated by acidic environments below pH 5, confirming and extending earlier reports on the effect

of pH on A β solubility (Burdick, D., *J. Biol. Chem.* 267:546-554 (1992)). Zn²⁺ (30 μ M) induced a constant level (~50%) of aggregation between pH 6.2-8.5, while below pH 6.0, aggregation could be explained solely by the effect of [H⁺].

In the presence of Cu²⁺ (30 μ M), a decrease in pH from 8.8 to 7.4 induced a marked drop in A β ₁₋₄₀ solubility, while a slight decrease below pH 7.4 strikingly potentiated the effect of Cu²⁺ on the peptide's aggregation. Surprisingly, Cu²⁺ caused >85 % of the available peptide to aggregate by pH 6.8, a pH which plausibly represents a mildly acidotic environment. Thus, conformational changes in A β brought about by small increases in [H⁺] result in the unmasking of a second metal binding site that leads to its rapid self-aggregation. Below pH 5.0, the ability of both Zn²⁺ and Cu²⁺ to aggregate A β was diminished, consistent with the fact that Zn binding to A β is abolished below pH 6.0 (Bush, A.I., *et al.*, *J. Biol. Chem.* 269:12152 (1994)), probably due to protonation of histidine residues.

The relationship between pH and Cu²⁺ on A β ₁₋₄₀ solubility was then further defined by the following experiments (FIG. 4B). The proportion of soluble A β ₁₋₄₀ remaining in the supernatant after incubation (30 min., 37 °C) at pH 5.4-7.8 with different Cu²⁺ concentrations (0, 5, 10, 20, 30 μ M), and centrifugation (10,000g, 20 min.), was measured and expressed as a percentage of starting peptide. All data points are means \pm SD, n=3. At pH 7.4, Cu²⁺-induced A β aggregation was 50% less than that induced by Zn²⁺ over the same concentration range, consistent with earlier reports (Bush, A.I., *et al.*, *J. Biol. Chem.* 269:12152 (1994)). There was a potentiating relationship between [H⁺] and [Cu²⁺] in producing A β aggregation; as the pH fell, less Cu²⁺ was required to induce the same level of aggregation, suggesting that [H⁺] is controlling Cu²⁺ induced A β ₁₋₄₀ aggregation.

To confirm that this reaction occurs at physiological concentrations of A β ₁₋₄₀ and Cu²⁺, a novel filtration immunodetection system was employed. This technique enabled the determination of the relative amount of A β ₁₋₄₀ aggregation in the presence of different concentrations of H⁺ and Cu²⁺ (FIG. 4C). The relative aggregation of nM concentrations of A β ₁₋₄₀ at pH 7.4 and pH 6.6 in the presence

of different Cu^{2+} concentrations (0, 0.1, 0.2, 0.5 μM) were determined by this method. Data represent mean reflectance values of immunoblot densitometry expressed as a ratio of the signal obtained when the peptide is treated in the absence of Cu^{2+} . All data points are means \pm SD, $n = 2$.

5 This sensitive technique confirmed that physiological concentrations of $\text{A}\beta_{1-40}$ are aggregated under mildly acidic conditions and that aggregation was greatly enhanced by the presence of Cu^{2+} at concentrations as low as 200 nM. Furthermore, as previously observed at higher $\text{A}\beta_{1-40}$ concentrations, a decrease in pH from 7.4 to 6.6 potentiated the effect of Cu^{2+} on aggregation of
10 physiological concentrations of $\text{A}\beta_{1-40}$. Thus, $\text{A}\beta_{1-40}$ aggregation is concentration independent down to 8 nM where Cu^{2+} is available.

 It has recently been shown that Zn^{2+} mediated $\text{A}\beta_{1-40}$ aggregation is reversible whereas $\text{A}\beta_{1-40}$ aggregation induced by pH 5.5 was irreversible (57). Therefore, we tested experiments were performed to determine whether Cu^{2+} /pH-mediated $\text{A}\beta_{1-40}$ aggregation was reversible. Cu^{2+} -induced $\text{A}\beta_{1-40}$ aggregation at
15 pH 7.4 was reversible following EDTA chelation, although for each new aggregation cycle, complete resolubilization of the aggregates required a longer incubation. This result suggested that a more complex aggregate is formed during each subsequent aggregation cycle, preventing the chelator access to
20 remove Cu^{2+} from the peptide. This is supported by the fact that complete resolubilization occurs with time, and indicates that the peptide is not adopting a structural conformation that is insensitive to Cu^{2+} -induced aggregation/EDTA-resolubilization.

 The reversibility of pH potentiated Cu^{2+} -induced $\text{A}\beta_{1-40}$ aggregation was
25 studied by turbidometry between pH 7.5 to 6.6, representing H^+ concentration extremes that might be found *in vivo* (FIGS. 5A and 5B). Unlike the irreversible aggregation of $\text{A}\beta_{1-40}$ observed at pH 5.5. Cu^{2+} -induced $\text{A}\beta_{1-40}$ aggregation was fully reversible as the pH oscillated between pH 7.4 and 6.6. Figure 5A shows the turbidometric analysis of Cu^{2+} -induced $\text{A}\beta_{1-40}$ aggregation at pH 7.4 reversed
30 by successive cycles of chelator (EDTA), as indicated. Figure 5B shows

turbidometric analysis of the reversibility of Cu^{2+} -induced $\text{A}\beta_{1-40}$ as the pH cycles between 7.4 and 6.6. Thus, subtle conformational changes within the peptide induced by changing $[\text{H}^+]$ within a narrow pH window, that corresponds to physiologically plausible $[\text{H}^+]$, allows the aggregation or resolubilization of the peptide in the presence of Cu^{2+} .

Discussion

These results suggest that subtle conformational changes in $\text{A}\beta$ induced by $[\text{H}^+]$ promote the interaction of $\text{A}\beta_{1-40}$ with metal ions, in particular Cu^{2+} and Hg^{2+} allowing self-aggregate or resolubilize depending on the $[\text{H}^+]$ (FIGs. 2A, 2B, 2C, 4A, 4B, 4C). A decrease in pH below 7.0 increases the β -sheet conformation (Soto, C., *et al.*, *J. Neurochem.* 63:1191-1198 (1994)), and this may allow the binding of Cu^{2+} to soluble $\text{A}\beta$ that could further alter the conformation of the peptide allowing for self aggregation, or simply help coordinate adjacent $\text{A}\beta$ molecules in the assembly of the peptides into aggregates. Conversely, increasing pH above 7.0 promotes the α -helical conformation (Soto, C., *et al.*, *J. Neurochem.* 63:1191-1198 (1994)), which may alter the conformational state of the dimeric aggregated peptide, releasing Cu and thereby destabilizing the aggregate with the resultant release of $\text{A}\beta$ into solution. Thus, in the presence of Cu^{2+} , $\text{A}\beta_{1-40}$ oscillates between an aggregated and soluble state dependent upon the $[\text{H}^+]$.

Interestingly, Hg^{2+} mediated $\text{A}\beta$ aggregation has greatly potentiated by mild acidity (FIG. 2C). $\text{A}\beta_{1-40}$ aggregation by Co^{2+} , like Zn^{2+} , was pH insensitive and per mole induced a similar level of aggregation. Unlike Zn^{2+} , $\text{A}\beta_{1-40}$ binding of Co^{2+} may be employed for the structural determination of the pH insensitive binding site given its nuclear magnetic capabilities.

The biphasic relationship of $\text{A}\beta$ solubility with pH mirrors the conformational changes previously observed by CD spectra within the N-terminal fragment (residues 1-28) of $\text{A}\beta$ (reviewed in (Soto, C., *et al.*, *J. Neurochem.*

63:1191-1198 (1994)); α -helical between pH 1-4 and >7, but β -sheet between pH 4-7. The irreversible aggregates of A β formed at pH 5.5 supports the hypothesis that the β -sheet conformation is a pathway for A β aggregation into amyloid fibrils. Since aggregates produced by Zn²⁺ and Cu²⁺ under mildly acidic conditions (FIGs. 5A and 5B) are chelator/pH reversible, there conformation may be the higher energy α -helical conformation.

These results now indicate that there are three physiologically plausible conditions which could aggregate A β : pH (FIGs. 1, 4A-4C; Fraser, P.E., *et al.*, *Biophys. J.* 60:1190-1201 (1991); Barrow, C.J. and Zagorski, M.G., *Science* 253:179-182 (1991); Burdick, D., *J. Biol. Chem.* 267:546-554 (1992); Barrow, C.J., *et al.*, *J. Mol. Biol.* 225:1075-1093 (1992); Zagorski, M.G. and Barrow, C.J., *Biochemistry* 31:5621-5631 (1992); Kirshenbaum, K. and Daggett, V., *Biochemistry* 34:7629-7639 (1995); Wood, S.J., *et al.*, *J. Mol. Biol.* 256:870-877 (1996), [Zn²⁺] (FIGs. 1, 2A and 2B, 4A-4C; Bush, A.I., *et al.*, *J. Biol. Chem.* 269:12152 (1994); Bush, A.I., *et al.*, *Science* 265:1464 (1994); Bush, A.I., *et al.*, *Science* 268:1921 (1995); Wood, S.J., *et al.*, *J. Mol. Biol.* 256:870-877 (1996)) and under mildly acidic conditions, [Cu²⁺] (FIGs. 2A, 4A-4C, 5B). Interestingly, changes in metal ion concentrations and pH are common features of the inflammatory response to injury. Therefore, the binding of Cu²⁺ and Zn²⁺ to A β may be of particular importance during inflammatory processes, since local sites of inflammation can become acidic (Trehaut, P.S. and McCarty, D.J., *Arthr. Rheum.* 14:475-484 (1971); Menkin, V., *Am. J. Pathol.* 10:193-210 (1934)) and both Zn²⁺ and Cu²⁺ are rapidly mobilized in response to inflammation (Lindeman, R.D., *et al.*, *J. Lab. Clin. Med.* 81:194-204 (1973); Terhune, M.W. and Sandstead, H.H., *Science* 177:68-69 (1972); Hsu, J.M., *et al.*, *J. Nutrition* 99:425-432 (1969); Haley, J.V., *J. Surg. Res.* 27:168-174 (1979); Milaninio, R., *et al.*, *Advances in Inflammation Research* 1:281-291 (1979); Frieden, E., in *Inflammatory Diseases and Copper*, Sorenson, J.R.J., ed, Humana Press, New Jersey (1980), pp. 159-169).

Serum copper levels increase during inflammation, associated with increases in ceruloplasmin, a Cu^{2+} transporting protein that may donate Cu^{2+} to enzymes active in processes of basic metabolism and wound healing such as cytochrome oxidase and lysyl oxidase (Giampaolo, V., *et al.*, in *Inflammatory Diseases and Copper*, Sorenson, J.R.J., ed, Humana Press, New Jersey (1980), pp. 329-345; Peacock, E.E. and vanWinkle, W., in *Wound Repair*, W.B. Saunders Co., Philadelphia (1976), pp. 145-155)). Since the release of Cu^{2+} from ceruloplasmin is greatly facilitated by acidic environments where the cupric ion is reduced to its cuprous form (Owen, C.A., Jr., *Proc. Soc. Exp. Biol. Med.* 149:681-682 (1975)), periods of mild acidosis may promote an environment of increased free Cu^{2+} . Similarly, aggregation of another amyloid protein, the acute phase reactant serum amyloid P component (SAP) to the cell wall polysaccharide, zymosan, has been observed with Cu^{2+} at acidic pH (Potempa, L.A., *et al.*, *Journal of Biological Chemistry* 260:12142-12147 (1985)). Thus, exchange of Cu^{2+} to $\text{A}\beta_{1-40}$ during times of decreased pH may provide a mechanism for altering the biochemical reactivity of the protein required by the cell under mildly acidic conditions. Such a function may involve alterations in the aggregation/adhesive properties (FIGS. 1-5B) or oxidative functions of $\text{A}\beta$ at local sites of inflammation.

While the pathogenic nature of $\text{A}\beta_{1-42}$ in AD is well described (Maury, C.P.J., *Lab. Investig.* 72:4-16 (1995); Multhaup, G., *et al.*, *Nature* 325:733-736 (1987)), the function of the smaller $\text{A}\beta_{1-40}$ remains unclear. The present data suggest that Cu^{2+} -binding and aggregation of $\text{A}\beta$ will occur when the pH of the microenvironment rises. This conclusion can be based on the finding that the reaction is $[\text{H}^+]$ and $[\text{Cu}^{2+}]$ dependent and reversible within a narrow, physiologically plausible, pH window. This is further supported by the specificity and high affinity of Cu^{2+} binding under mildly acidic conditions compared to the constant Zn^{2+} -induced aggregation (and binding) of $\text{A}\beta_{1-40}$ over a wide pH range (6.2-8.5). The brain contains high levels of both Zn^{2+} ($\sim 150 \mu\text{M}$; Frederickson, C.J. *International Review of Neurobiology* 31:145-237 (1989)) and Cu^{2+} (~ 100

5 μM ; Warren, P.J., *et al.*, *Brain* 83:709-717 (1960); Owen, C.A., *Physiological Aspects of Copper*, Noyes Publications, Park Ridge, New Jersey (1982), pp160-191). Intracellular concentrations are approximately 1000 and 100 fold higher than extracellular concentrations. This large gradient between intracellular and extracellular compartments suggests a highly energy dependent mechanism is required in order to sequester these metals within neurons. Therefore, any alterations in energy metabolism, or injury, may affect the reuptake of these metal ions and promote their release into the extracellular space, and together with the synergistic effects of decreased pH (see above) induce membrane bound $\text{A}\beta_{1-40}$ to aggregate. Since increased concentrations of Zn^{2+} and Cu^{2+} , and decreased pH, are common features of all forms of cellular insult, the initiation of $\text{A}\beta_{1-40}$ function likely occurs in a coordinated fashion to alter adhesive and/or oxidative properties of this membrane protein essential for maintaining cell integrity and viability. That $\text{A}\beta_{1-40}$ has such a high affinity for these metal ions, indicates a protein that has evolved to respond to slight changes in the concentration of extracellular metal ions. This is supported by the fact that aggregation in the presence of Cu is approx. 30% at pH 7.1, the pH of the brain (Yates CM, *et al.*, *J. Neurochem.* 55:1624-1630 (1990)), but 85% at pH 6.8. Taken together, our present results indicate that $\text{A}\beta_{1-40}$ may have evolved to respond to biochemical changes associated with neuronal damage as part of the locally mediated response to inflammation or cell injury. Thus, it is possible that Cu^{2+} mediated $\text{A}\beta_{1-40}$ binding and aggregation might be a purposive cellular response to an environment of mild acidosis.

25 The deposition of amyloid systemically is usually associated with an inflammatory response (Pepys, M.B. and Baltz, M.L., *Adv. Immunol.* 34:141-212 (1983); Cohen, A.S., in *Arthritis and Allied Conditions*, D.J. McCarty, ed., Lea and Febiger, Philadelphia (1989), pp. 1273-1293; Kisilevsky, R., *Lab. Investig.* 49:381-390 (1983)). For example, serum amyloid A, one of the major acute phase reactant proteins that is elevated during inflammation, is the precursor of amyloid A protein that is deposited in various tissues during chronic

30

inflammation, leading to secondary amyloidosis (Gorevic, P.D., *et al.*, *Ann. NY Acad. Sci.*:380-393 (1982)). An involvement of inflammatory mechanisms has been suggested as contributing to plaque formation in AD (Kisilevsky, R., *Mol. Neurobiol.* 49:65-66 (1994)). Acute-phase proteins such as alpha 1-antichymotrypsin and c-reactive protein, elements of the complement system and activated microglial and astroglial cells are consistently found in AD brains.

The rapid appearance, within days of A β deposits and APP immunoreactivity following head injury (Roberts, G. W., *et al.*, *Lancet.* 338:1422-1423 (1991); Pierce, J.E.S., *et al.*, *Journal of Neuroscience* 16:1083-1090 (1996)), rather than the more gradual accumulation of A β into more dense core amyloid plaques over months or years in AD may be compatible with the release of Zn²⁺, Cu²⁺ and mild acidosis in this time frame. Thus, pH/metal ion mediated aggregation may form the basis for the amorphous A β deposits observed in the aging brain and following head injury, allowing the maintenance of endothelial and neuronal integrity while limiting the oxidative stress associated with injury that may lead to a diminishment of structural function.

Since decreased cerebral pH is a complication of aging (Yates CM, *et al.*, *J. Neurochem.* 55:1624-1630 (1990)), these data indicate that Cu and Zn mediated A β aggregation may be a normal cellular response to an environment of mild acidosis. However, prolonged exposure of A β to an environment of lowered cerebral pH may promote increased concentrations of free metal ions and reactive oxygen species, and the inappropriate interaction of A β ₁₋₄₂ over time promoting the formation of irreversible A β oligomers and its subsequent deposition as amyloid in AD. The reversibility of this pH mediated Cu²⁺ aggregation does however present the potential for therapeutic intervention. Thus, cerebral alkalization may be explored as a therapeutic modality for the reversibility of amyloid deposition *in vivo*.

Example 2

Free Radical Formation and SOD-like activity of Alzheimer's A β Peptides

a) *Determination of Cu⁺ and Fe²⁺*

This method is modified from a protocol assaying serum copper and iron (Landers, J.W. and Zak, B., *Chim. Acta.* 29:590 (1958)). It is based on the fact that there are optimal visible absorption wavelengths of 483 nm and 535 nm for Cu⁺ complexed with bathocuproinedisulfonic (BC) anion and Fe²⁺ coordinated by bathophenanthrolinedisulfonic (BP) anion, respectively.

Determination of molar absorption of these two complexes was accomplished essentially as follows. An aliquot of 500 μ l of each complex (500 μ M, in PBS pH 7.4, with ligands in excess) was pipetted into 1 cm-pathlength quartz cuvette, and their absorbances were measured. Their molar absorbancy was determined based on Beer-Lambert's Law. Cu⁺-BC has a molar absorbancy of 2762 M⁻¹ cm⁻¹, while Fe²⁺-BP has a molar absorbancy of 7124 M⁻¹ cm⁻¹.

Determination of the equivalent vertical pathlength for Cu⁺-BC and Fe²⁺-BP in a 96-well plate was carried out essentially as follows. Absorbances of the two complexes with a 500 μ M, 100 μ M, 50 μ M, and 10 μ M concentration of relevant metal ions (Cu⁺, Fe²⁺) were determined both by 96-well plate readers (300 μ L) and UV-vis spectrometer (500 μ L), with PBS, pH 7.4, as the control blank. The resulting absorbancies from the plate reader regress against absorbancies by a UV-vis spectrometer. The slope k from the linear regression line is equivalent to the vertical pathlength if the measurement is carried out on a plate. The results are:

	k(cm)	r ²
Cu ⁺ -BC	1.049	0.998
Fe ²⁺ -BP	0.856	0.999

With molar absorptivity and equivalent vertical pathlength in hand, the concentrations (μM) of Cu^+ or Fe^{2+} can be deduced based on Beer-Lambert's Law, using proper buffers as controls.

$$\text{for } \text{Cu}^+: [\text{Cu}^+] (\mu\text{M}) = \frac{\Delta A(483\text{nm})}{(2762 \times 1.049)} \times 10^6$$

$$\text{for } \text{Fe}^{2+}: [\text{Fe}^{2+}] (\mu\text{M}) = \frac{\Delta A (535\text{nm})}{(7124 \times 0.856)} \times 10^6$$

where ΔA is absorbance difference between sample and control blank.

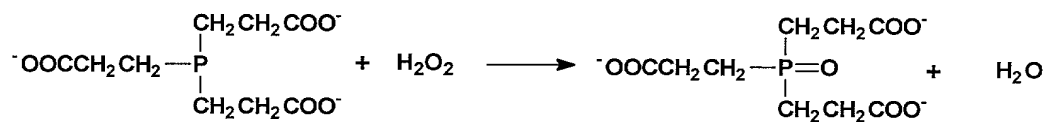
b) **Determination of H_2O_2**

This method is modified from a H_2O_2 assay reported recently (Han, J.C. *et al.*, *Anal. Biochem.* 234:107 (1996)). The advantages of this modified H_2O_2 assay on 96-well plate include high throughput, excellent sensitivity ($\sim 1 \mu\text{M}$), and the elimination of the need for a standard curve of H_2O_2 , which is problematic due to the labile chemical property of H_2O_2 .

$\text{A}\beta$ peptides were co-incubated with a H_2O_2 -trapping reagent (Tris(2-carboxyethyl)-phosphine hydrochloride, TCEP, $100 \mu\text{M}$) in PBS (pH 7.4 or 7.0) at 37°C for 30 mins. Then 5,5'-dithio-bis(2-nitrobenzoic acid) (DBTNB, $100 \mu\text{M}$) was added to react with remaining TCEP. The product of this reaction has a characteristic absorbance maximum of 412 nm [18]. The assay was adapted to a 96-well format using a standard absorbance range (see FIG. 11).

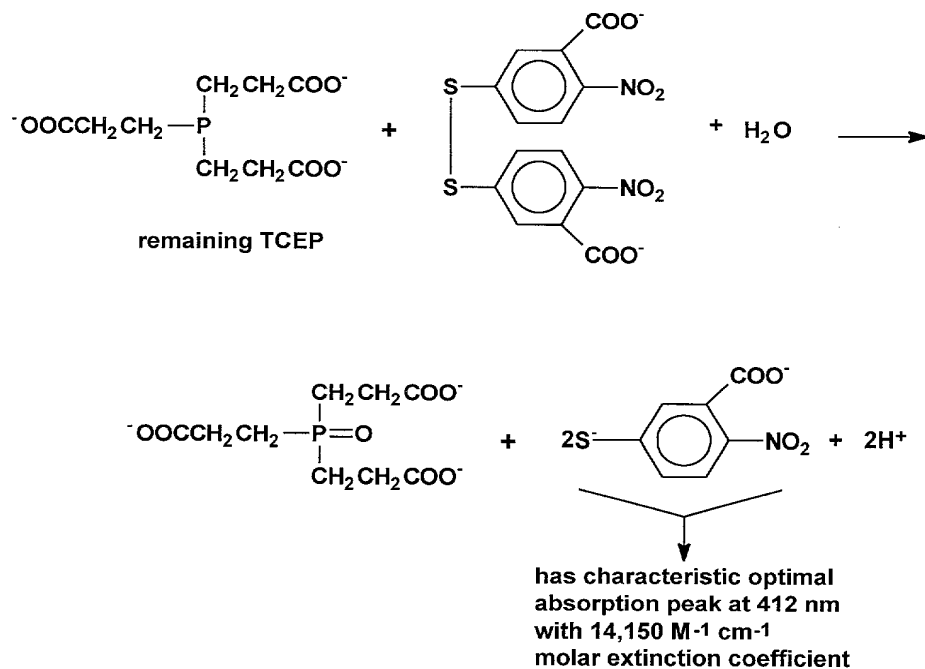
The chemical scheme for this novel method is:

Scheme I:

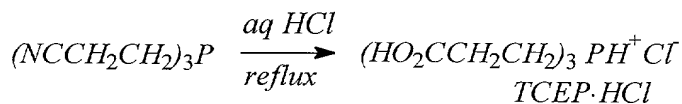


(TCEP) [Tris (2-carboxethyl) phosphine]

Scheme II:



TCEP•HCl was synthesized by hydrolyzing tris (2-cyno-ethyl) phosphine (purchased from Johnson-Mathey (Waydhill, MA)), in refluxing aqueous HCl (Burns, J.A. *et al.*, *J. Org. Chem.* 56:2648 (1991)) as shown below.



In order to carry out the above-described assay in a 96-well plate, it was necessary to calculate the equivalent vertical pathlength of 2-nitro-5-thiobenzoic acid (TMB) in a 96-well plate. This determination was carried out essentially as described for Cu⁺-BC and Fe²⁺-BP in Example 5. The resulting absorbancies from the plate reader regress against absorbancies by a UV-vis spectrometer. The slope k from the linear regression line is equivalent to the vertical pathlength if the measurement is carried out on a plate. The results are:

k	r ²
0.875	1.00

The concentration of H₂O₂ can then be deduced from the difference in absorbance between the sample and the control (sample plus 1000 U/μl catalase)

$$[H_2O_2] (\mu M) = \frac{\Delta A (412nm)}{(2 \times 0.875 \times 14150)}$$

c) Determination of OH[•]

Determination of OH[•] was performed as described in Gutteridge *et al.* (*Biochim. Biophys. Acta* 759: 38-41 (1983)).

d) Cu(I) generation by Aβ: influence of Zn(II) and pH

Aβ (10 μM in PBS, pH 7.4 or 6.8, as shown) was incubated for 30 minutes (37°C) in the presence of Cu(II) 10 μM ± Zn(II) 10 μM). Cu(I) levels (n=3, ±SD) were assayed against a standard curve. These data indicate that the presence of Zn(II) can mediate the reduction of Cu(II) in a mildly acidic environment. The effects of zinc upon these reactions are strongly in evidence

but complex. Since the presence of 10 μM zinc will precipitate the peptide, it is clear that the peptide possesses redox activity even when it is not in the soluble phase, suggesting that cortical A β deposits will not be inert in terms of generating these highly reactive products. Cerebral zinc metabolism is deregulated in AD, and therefore levels of interstitial zinc may play an important role in adjusting the Cu(I) and H₂O₂ production generated by A β . The rat homologue of A β ₁₋₄₀ does not manifest the redox reactivity of the human equivalent. Insulin, a histidine-containing peptide that can bind copper and zinc, exhibits no Cu(II) reduction.

e) Hydrogen peroxide production by A β species

AB₁₋₄₂ (10 μM) was incubated for 1 hr at 37°C, pH 7.4 in ambient air (first bar), continuous argon purging (Ar), continuous oxygen enrichment (O₂) at pH 7.0 (7.0), or in the presence of the iron chelator desferioxamine (220 μM ; DFO). Variant A β species (10 μM) were tested: A β ₁₋₄₀ (A β ₁₋₄₀), rat A β ₁₋₄₀ (rA β ₁₋₄₀), and scrambled A β ₁₋₄₀ (sA β ₁₋₄₀) were incubated for 1 hr at 37°C, pH 7.4 in ambient air. Values (mean \pm SD, n=3) represent triplicate samples minus values derived from control samples run under identical conditions in the presence of catalase (10 U/ml). The details of the experiment are as follows: A β peptides were co-incubated with a H₂O₂-trapping reagent (Tris(2-carboxyethyl)-phosphine hydrochloride, TCEP, 100 μM) in PBS (pH 7.4 or 7.0) at 37°C for 30 mins. Then 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB, 100 μM) was added to react with remaining TCEP, the product has a characteristic absorbance maximum of 412 nm. The assay was adapted to a 96-well format using a standard absorbance range.

Results and Discussion

A β exhibits metal-dependent and independent redox activity

Because A β was observed to be covalently linked by Cu, the ability of the peptide to reduce metals and generate hydroxyl radicals was studied. The bathocuproine and bathophenanthroline reduced metal assay technique employed by Multhaup *et al.* was used in order to determine that APP itself possesses a Cu(II) reducing site on its ectodomain (Multhaup, G., *et al.*, *Science* 271:1406 (1996)). It has been discovered that A β possesses a striking ability to reduce both Fe(III) to Fe(II), and Cu(II) to Cu(I), modulated by Zn(II) and pH (6.6-7.4) (FIG. 10). It is of great interest that the relative redox activity of the peptides studied correlates so well with their relative pathogenicity viz A β 42>A β 40>ratA β in all redox assays studied. Since one of the caveats in using the reduced metals assay is that the detection agents can exaggerate the oxidation potential of Cu(II) or Fe (III), other redox products were explored by assays where no metal ion indicators were necessary. It was discovered that hydrogen peroxide was rapidly formed by A β species (FIG. 11). Thus, A β produces both H₂O₂ and reduced metals whilst also binding zinc. Structurally, this is difficult to envisage for a small peptide, but we have recently shown that A β is dimeric in physiological buffers. Since H₂O₂ and reduced metal species are produced in the same vicinity, these reaction products are liable to produce the highly toxic hydroxyl radical by Fenton chemistry, and the formation of hydroxyl radicals from these peptides has now been shown with the thiobarbituric acid assay. The formation of hydroxyl radicals correlates with the covalent polymerization of the peptide (FIG. 9) and can be blocked by hydroxyl scavengers. Thus the concentrations of Fe, Cu, Zn & H⁺ in the brain interstitial milieu could be important in facilitating precipitation and neurotoxicity for A β by direct (dimer formation) and indirect (Fe(II)/Cu(I) and H₂O₂ formation) mechanisms.

H₂O₂ production by A β explains the mechanism by which H₂O₂ has been described to mediate neurotoxicity (Behl, C., *et al.*, *Cell* 77:827 (1994)),

previously thought to be the product of cellular overproduction alone. Interestingly, the scrambled A β peptide produces appreciable H₂O₂ (FIG. 6) but no hydroxyl radicals. This is because the scrambled A β peptide is unable to reduce metal ions. Therefore, we conclude that what makes A β such a potent neurotoxin is its capacity to produce both reduced metal and H₂O₂ at the same time. This "double whammy" can then produce hydroxyl radicals by the Fenton reaction, especially if the H₂O₂ is not rapidly removed from the vicinity of the peptide. Catalase and glutathione peroxidase are the principal means of catabolizing H₂O₂, and their levels are low in the brain, especially in AD, perhaps explaining the propensity of A β to accumulate in brain tissue.

FIG. 11 shows that the production of H₂O₂ is oxygen dependent, and further investigation has indicated that A β can spontaneously produce the superoxide radical (O₂⁻) in the absence of metal ions. This property of A β is particularly exaggerated in the case of A β 42, probably explaining why this peptide is more neurotoxic and more enriched than A β 40 in amyloid. O₂⁻ generation will be subject to spontaneous dismutation to generate H₂O₂, however, this is a relatively slow reaction, although it may account for the majority of the H₂O₂ detected in our A β assays. O₂⁻ is reactive, and the function of superoxide dismutase (SOD) is to accelerate the dismutation to produce H₂O₂ which is then catabolized by catalase and peroxidases into oxygen and water. The most abundant form of SOD is Cu/Zn SOD, mutations of which cause another neurodegenerative disease, amyotrophic lateral sclerosis (Rosen, D., *et al.*, *Nature* 364:362 (1993)). Since A β , like Cu/Zn SOD, is a dimeric protein that binds Cu and Zn and reduces Cu(II) and Fe(III), we studied the O₂⁻ dismutation behavior of A β in the μ sec time-scale using laser pulse photolysis. These experiments have shown that A β exhibits Fe/Cu-dependent SOD-like activity with rate constants of dismutation at $\approx 10^8$ M⁻¹ sec⁻¹, which are strikingly similar to SOD. Hence, A β appears to be a good candidate to possess the same function as SOD, and therefore may function as an antioxidant. This may explain why oxidative stresses cause it to be released by cells (Frederikse, P.H., *et al.*, *Journal of*

Biological Chemistry 271: 10169 (1996)). However, if A β ₁₋₄₂ is involved in the reaction to oxidative stress, or if the H₂O₂ clearance is compromised at the cellular level, A β will accumulate, recruiting more O₂ and producing more O₂⁻ leading to a vicious cycle and localizing tissue Peroxidation damage and protein cross-linking.

Example 3

Cell Culture and Cytotoxic Assays

Several different assays may be utilized to determine whether a candidate pharmacological agent identified by any of the above-summarized assays is capable of altering the neurotoxicity of A β . The first is the MTT assay, which measures the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyl tetrazolium bromide (MTT) to a colored formazon (Hansen *et al.*, 1989). A second cytotoxic assay is the release of lactic dehydrogenase (LDH) from cells, a measurement routinely used to quantitate cytotoxicity in cultured CNS cells (Choi, 1987). While MTT measures primarily early redox changes within the cell reflecting the integrity of the electron transport chain, the release of LDH is thought to be through cell lysis. A third assay is visual counting in conjunction with trypan blue exclusion. Yet another assay is the Live/Dead EukoLight Viability/Cytotoxicity Assay (Molecular Probes, Inc., Eugene, OR).

Having now fully described this invention, it will be understood by those of skill in the art that it can be performed within any wide range of equivalent modes of operation as well as other parameters without affecting the scope of the invention or any embodiment thereof.

All patents and publications cited in the present specification are incorporated by reference herein in their entirety.

What Is Claimed Is:

1. A method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of altering the production of Cu(I) by A β , said method comprising:

- (a) adding Cu(II) to a first A β sample;
 - (b) allowing said first sample to incubate for an amount of time sufficient to allow said first sample to generate Cu(I);
 - (c) adding Cu(II) to a second A β sample, said second sample additionally comprising a candidate pharmacological agent;
 - (d) allowing said second sample to incubate for the same amount of time as said first sample;
 - (e) determining the amount of Cu(I) produced by said first sample and said second sample; and
 - (f) comparing the amount of Cu(I) produced by said first sample to the amount of Cu(I) produced by said second sample;
- whereby a difference in the amount of Cu(I) produced by said first sample as compared to said second sample indicates that said candidate pharmacological agent has altered the production of Cu(I) by A β .

2. The method of claim 1, wherein the amount of Cu(I) present in said first and said second sample is determined by

- (a) adding a complexing agent to said first and said second sample, wherein said complexing agent is capable of combining with Cu(I) to form a complex compound, wherein said complex compound has an optimal visible absorption wavelength;
- (b) measuring the absorbancy of said first and said second sample; and
- (c) calculating the concentration of Cu(I) in said first and said second sample using the absorbancy obtained in step (b).

3. The method of claim 2, wherein said complexing agent is bathocuproinedisulfonic anion.

4. The method of claim 2 or claim 3, wherein said method is performed in a microtiter plate, and the absorbancy measurement is performed by a plate reader.

5. The method of claim 4, wherein two or more different test candidate agents are simultaneously evaluated for an ability to alter the production of Cu(I) by A β .

6. The method of claim 1, wherein said first A β sample of step 1(a) and said second A β sample of step 1(c) is a biological sample.

7. The method of claim 6, wherein said biological sample is CSF.

8. A method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of altering the production of Fe(II) by A β , said method comprising:

- (a) adding Fe(III) to a first A β sample;
- (b) allowing said first sample to incubate for an amount of time sufficient to allow said first sample to generate Fe(II);
- (c) adding Fe(III) to a second A β sample, said second sample additionally comprising a candidate pharmacological agent;
- (d) allowing said second sample to incubate for the same amount of time as said first sample;
- (e) determining the amount of Fe(II) produced by said first sample and said second sample; and
- (f) comparing the amount of Fe(II) present in said first sample to the amount of Fe(II) present in said second sample;

whereby a difference in the amount of Fe(II) present in said first sample as compared to said second sample indicates that said candidate pharmacological agent has altered the production of Fe(II) by A β .

9. The method of claim 8, wherein the amount of Fe(II) present in said first and said second sample is determined by

(a) adding a complexing agent to said first and said second sample, wherein said complexing agent is capable of combining with Fe(II) to form a complex compound, wherein said complex compound has an optimal visible absorption wavelength;

(b) measuring the absorbancy of said first and said second sample; and

(c) calculating the concentration of Fe(II) in said first and said second sample using the absorbancy obtained in step (b).

10. The method of claim 9, wherein said complexing agent is bathophenanthrolinedisulfonic (BP) anion.

11. The method of claim 9 or claim 10, wherein said method is performed in a microtiter plate, and the absorbancy measurement is performed by a plate reader.

12. The method of claim 11, wherein two or more different test candidate agents are simultaneously evaluated for an ability to alter the production of Fe(II) by A β .

13. The method of claim 8, wherein said first A β sample of step 1(a) and said second A β sample of step 1(c) is a biological sample.

14. The method of claim 13, wherein said biological sample is CSF.

15. A method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of altering the production of H_2O_2 by $A\beta$, said method comprising:

- (a) adding Cu(II) or Fe(III) to a first $A\beta$ sample;
 - (b) allowing said first sample to incubate for an amount of time sufficient to allow said first sample to generate H_2O_2 ;
 - (c) adding Cu(II) or Fe(III) to a second $A\beta$ sample, said second sample additionally comprising a candidate pharmacological agent;
 - (d) allowing said second sample to incubate for the same amount of time as said first sample;
 - (e) determining the amount of H_2O_2 produced by said first sample and said second sample; and
 - (f) comparing the amount of H_2O_2 present in said first sample to the amount of H_2O_2 present in said second sample;
- whereby a difference in the amount of H_2O_2 present in said first sample as compared to said second sample indicates that said candidate pharmacological agent has altered the production of H_2O_2 by $A\beta$.

16. The method of claim 15, wherein the $A\beta$ samples of steps (a) and step (b) are a biological fluid.

17. The method of claim 16, wherein said biological fluid is CSF.

18. The method of claim 15, wherein the determination of step (e) of the amount of H_2O_2 present in said first and said second sample is determined by

- (a) adding catalase to a first aliquot of said first sample obtained in step (a) of claim 1 in an amount sufficient to break down all of the H_2O_2 generated by said sample;
- (b) adding TCEP, in an amount sufficient to capture all of the H_2O_2 generated by said samples, to

- (i) said first aliquot
- (ii) a second aliquot of said first sample obtained in step (a) of claim 1; and
- (iii) said second sample obtained in step (b) of claim 1;
- (c) incubating the samples obtained in step (b) for an amount of time sufficient to allow the TCEP to capture all of the H_2O_2 ;
- (d) adding DTNB to said samples obtained in step (c);
- (e) incubating said samples obtained in step (d) for an amount of time sufficient to generate TMB;
- (f) measuring the absorbancy at 412 nm of said samples obtained in step (e); and
- (g) calculating the concentration of H_2O_2 in said first and said second sample using the absorbancies obtained in step (f).

19. The method of claim 18, wherein said method is performed in a microtiter plate, and the absorbancy measurement is performed by a plate reader.

20. The method of claim 19, wherein two or more different test candidate agents are simultaneously evaluated for an ability to alter the production of H_2O_2 by $A\beta$.

21. A method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of decreasing the production of O_2^- by $A\beta$, said method comprising:

- (a) adding $A\beta$ and to a first buffer sample having an O_2 tension greater than 0;
- (b) allowing said first sample to incubate for an amount of time sufficient to allow said first sample to generate O_2^- ;
- (c) adding $A\beta$ and a candidate pharmacological agent to a second buffer sample having an O_2 tension greater than 0;;

(d) allowing said second sample to incubate for the same amount of time as said first sample;

(e) determining the amount of O_2^- produced by said first sample and said second sample; and

(f) comparing the amount of O_2^- present in said first sample to the amount of O_2^- present in said second sample;

whereby a difference in the amount of O_2^- present in said first sample as compared to said second sample indicates that said candidate pharmacological agent has altered the production of O_2^- by $A\beta$.

22. The method of claim 21, wherein said $A\beta$ is $A\beta_{1-42}$.

23. The method of claim 21, wherein the determination of the amount of O_2^- present in said samples is accomplished by measuring the absorbancy of the sample at about 250 nm.

24. A method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of interfering with the interaction of O_2 and $A\beta$ to produce O_2^- , without interfering with the SOD-like activity of $A\beta$, said method comprising:

(a) identifying an agent capable of decreasing the production of O_2^- by $A\beta$; and

(b) determining the ability of said agent to alter the SOD-like activity of $A\beta$.

25. The method of claim 24, wherein the determination of the ability of said agent to alter the SOD-like activity of $A\beta$ is made by determining whether $A\beta$ is capable of catalytically producing $Cu(I)$, $Fe(II)$ or H_2O_2 .

26. A method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of reducing the toxicity of A β , said method comprising:

- (a) adding A β to a first cell culture;
- (b) adding A β to a second cell culture, said second cell culture additionally containing a candidate pharmacological agent;
- (c) determining the level of neurotoxicity of A β in said first and said second samples; and
- (d) comparing the level of neurotoxicity of A β in said first and said second samples,

whereby a lower neurotoxicity level in said second sample as compared to said first sample indicates that said candidate pharmacological agent has reduced the neurotoxicity of A β , and is thereby capable of being used to treat AD.

27. The method of claim 26, wherein the neurotoxicity of A β is determined by using an MTT assay.

28. The method of claim 26, wherein the neurotoxicity of A β is determined by using an LDH release assay.

29. The method of claim 26, wherein the neurotoxicity of A β is determined by using a Live/Dead assay.

30. The method of claim 26, wherein said cells are rat cancer cells.

31. The method of claim 26, wherein said cells are rat primary frontal neuronal cells.

32. A kit for determining whether an agent is capable of altering the production of Cu(I) by A β which comprises a carrier means being

compartmentalized to receive in close confinement therein one or more container means wherein

- (a) the first container means contains a peptide comprising A β peptide;
- (b) a second container means contains a Cu(II) salt; and
- (c) a third container means contains BC anion.

33. The kit of claim 32, wherein said A β peptide is present as a solution in an aqueous buffer or a physiological solution, at a concentration above about 10 μ M.

34. A kit for determining whether an agent is capable of altering the production of Fe(II) by A β which comprises a carrier means being compartmentalized to receive in close confinement therein one or more container means wherein

- (a) the first container means contains a peptide comprising A β peptide;
- (b) a second container means contains an Fe(III) salt; and
- (c) a third container means contains BP anion.

35. The kit of claim 34, wherein said A β peptide is present as a solution in an aqueous buffer or a physiological solution, at a concentration above about 10 μ M.

36. A kit for determining whether an agent is capable of altering the production of H₂O₂ by A β which comprises a carrier means being compartmentalized to receive in close confinement therein one or more container means wherein

- (a) the first container means contains a peptide comprising A β peptide;

- (b) a second container means contains a Cu(II) salt;
- (c) a third container means contains TCEP; and
- (d) a fourth container means contains DTNB.

37. The kit of claim 36, wherein said A β peptide is present as a solution in an aqueous buffer or a physiological solution, at a concentration above about 10 μ M.

38. A method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of inhibiting redox-reactive metal-mediated crosslinking A β , said method comprising:

- (a) adding a redox-reactive metal to a first A β sample;
- (b) allowing said first sample to incubate for an amount of time sufficient to allow A β crosslinking;
- (c) adding said redox-reactive metal to a second A β sample, said second sample additionally comprising a candidate pharmacological agent;
- (d) allowing said second sample to incubate for the same amount of time as said first sample;
- (e) removing an aliquot from each of said first and said second sample; and
- (f) determining presence or absence of crosslinking in said first and second samples,

whereby an absence of A β crosslinking in said second sample as compared to said first sample indicates that said candidate pharmacological agent has inhibited A β crosslinking.

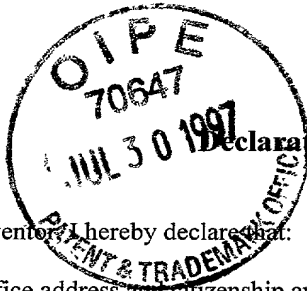
39. The method of claim 38, wherein at step (f), a western blot analysis is performed to determine the presence or absence of crosslinking in the first and the second sample.

Methods for the Identification of Agents for Use in the Treatment of Alzheimer's Disease

Abstract

5 The invention relates to methods for the identification of candidate pharmacological agents to be used in the treatment of Alzheimer's disease and related pathological conditions.

A99-01.WPD



Declaration for Patent Application

Docket Number: 0609.4350000

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter that is claimed and for which a patent is sought on the invention entitled Method for the Identification of Agents for Use in the Treatment of Alzheimer's Disease, the specification of which is attached hereto unless the following box is checked:

- ☒ was filed on March 11, 1997 ;
as United States Application Number or PCT International Application Number 08/816,122 ; and
was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application, which designated at least one country other than the United States listed below, and have also identified below any foreign application for patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Claimed
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No
(Application No.)	(Country)	(Day/Month/Year Filed)	
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No
(Application No.)	(Country)	(Day/Month/Year Filed)	

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

_____	_____
(Application No.)	(Filing Date)
_____	_____
(Application No.)	(Filing Date)

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or under § 365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56 that became available between the filing date of the prior application and the national or PCT international filing date of this application.

_____	_____	_____
(Application No.)	(Filing Date)	(Status - patented, pending,
abandoned)		
_____	_____	_____
(Application No.)	(Filing Date)	(Status - patented, pending,
abandoned)		

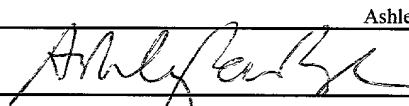
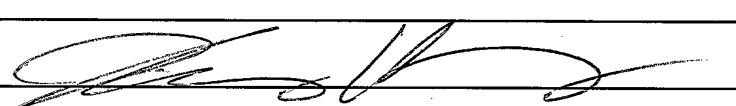
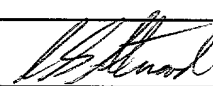
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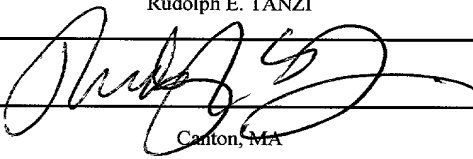
STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.
1100 New York Avenue, N.W.
Suite 600
Washington, D.C. 20005-3934

Direct Telephone Calls to:

(202) 371-2600

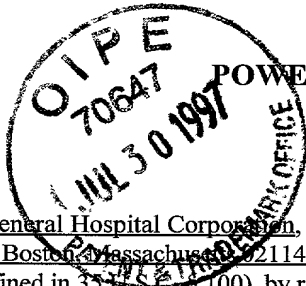
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor	Ashley I. BUSH
Inventor's signature	 7/11/97 Date
Residence	91 SUMMER STREET #3, Somerville, MA 02143
Citizenship	AUSTRALIAN
Post Office Address	91 Summer St., #3
	Somerville, MA 02143
Full name of second inventor	Xudong HUANG
Second Inventor's signature	 Date
Residence	Cambridge, MA
Citizenship	Chinese
Post Office Address	402 Range Ave., Apt. 10L
	Cambridge, MA 02140
Full name of third inventor	Craig S. Atwood
Third inventor's signature	 Date 6/25/97
Residence	Somerville, MA
Citizenship	Australian
Post Office Address	19 Aldersen St., #3
	Somerville, MA 02143

Full name of fourth inventor	Rudolph E. TANZI
Fourth inventor's signature	 Date
Residence	Canton, MA
Citizenship	American
Post Office Address	25 Strawberry Ln.
	Canton, MA 02021
Full name of fifth inventor	
Fifth inventor's signature	Date
Residence	
Citizenship	
Post Office Address	

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SKGF Rev 4/3/96

(Supply similar information and signature for subsequent joint inventors, if any)



POWER OF ATTORNEY FROM ASSIGNEE

The General Hospital Corporation, a corporation of Massachusetts, having a principal place of business at Fruit Street, Boston, Massachusetts 02114, is assignee of the entire right, title and interest for the United States of America (as defined in 35 U.S.C. § 100), by reason of an Assignment to the Assignee executed on July 11, June 25, June 25 and June 25, 1997 of an invention known as Method for the Identification of Agents for Use in the Treatment of Alzheimer's Disease (Attorney Docket No. 0609.4350000), which is disclosed and claimed in a patent application of the same title by the inventors (1) Ashley I. BUSH, (2) Xudong HUANG, (3) Craig S. ATWOOD and (4) Rudolph E. TANZI (said application filed on March 11, 1997 at the U.S. Patent and Trademark Office, having Application Number 08/816,122).

respectively
RUE
Reg No
32,893

The Assignee hereby appoints the following U.S. attorneys to prosecute this application and any continuation, divisional, continuation-in-part, or reissue application thereof, and to transact all business in the U.S. Patent and Trademark Office connected therewith: Robert Greene Sterne, Registration No. 28,912; Edward J. Kessler, Registration No. 25,688; Jorge A. Goldstein, Registration No. 29,021; Samuel L. Fox, Registration No. 30,353; David K.S. Cornwell, Registration No. 31,944; Robert W. Esmond, Registration No. 32,893; Tracy-Gene G. Durkin, Registration No. 32,831; Michele A. Cimbala, Registration No. 33,851; Michael B. Ray, Registration No. 33,997; Robert E. Sokohl, Registration No. 36,013; Eric K. Steffe, Registration No. 36,688; and Andrea G. Reister, Registration No. 36,253. The Assignee hereby grants said attorneys the power to insert on this Power of Attorney any further identification that may be necessary or desirable in order to comply with the rules of the U.S. Patent and Trademark Office.

Send correspondence to:

Sterne, Kessler, Goldstein & Fox P.L.L.C.
1100 New York Avenue, N.W.
Suite 600
Washington, D.C. 20005-3934
U.S.A.

Direct phone calls to 202-371-2600.

FOR: The General Hospital Corporation

SIGNATURE: 

BY: DAVID J. GLASS, Ph.D.

ASSOCIATE DIRECTOR FOR PATENTS
OFFICE OF TECHNOLOGY AFFAIRS

TITLE:

DATE: 7/11/97

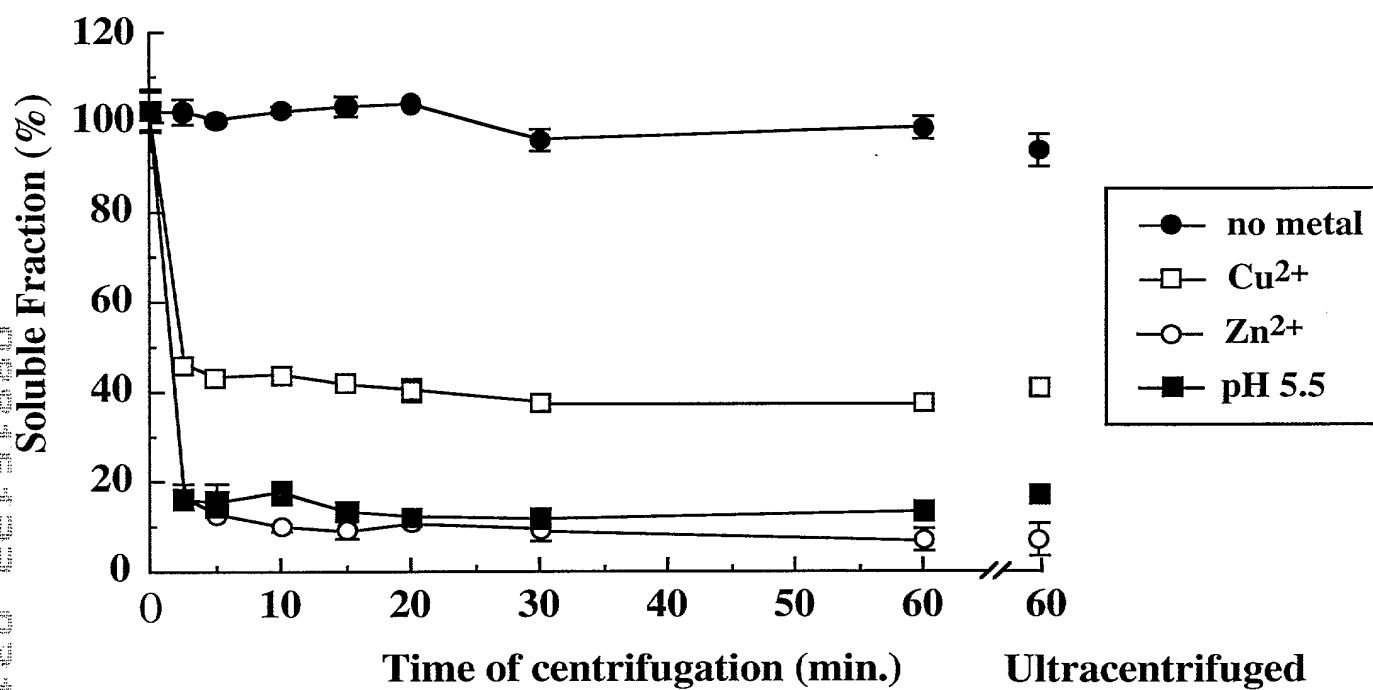


Fig 1.

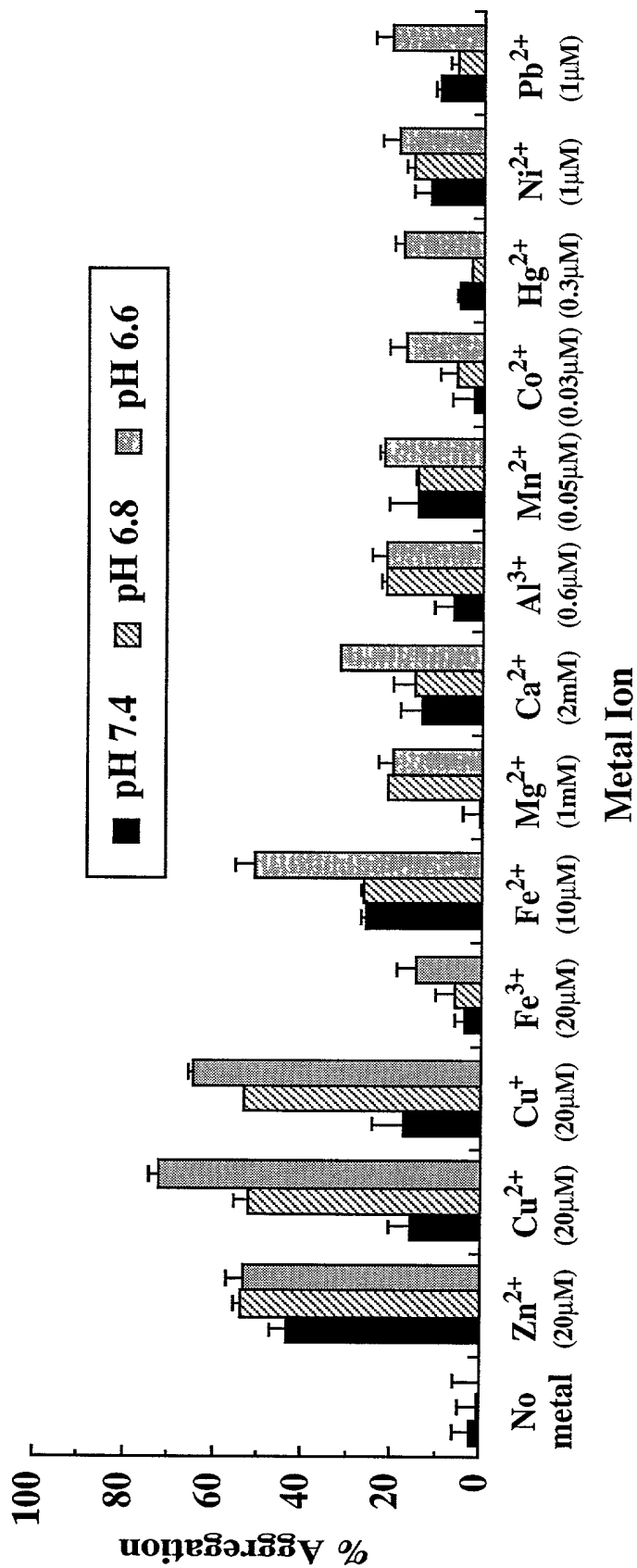


Fig 2a.

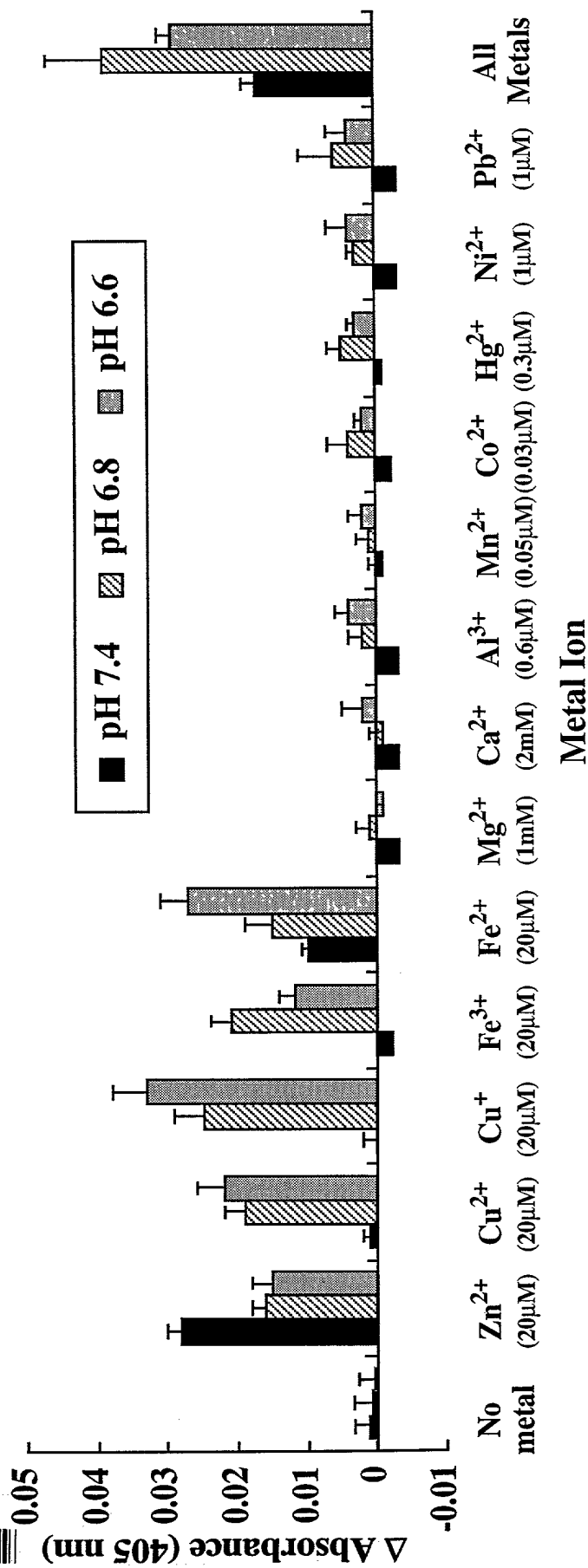


Fig 2 b.

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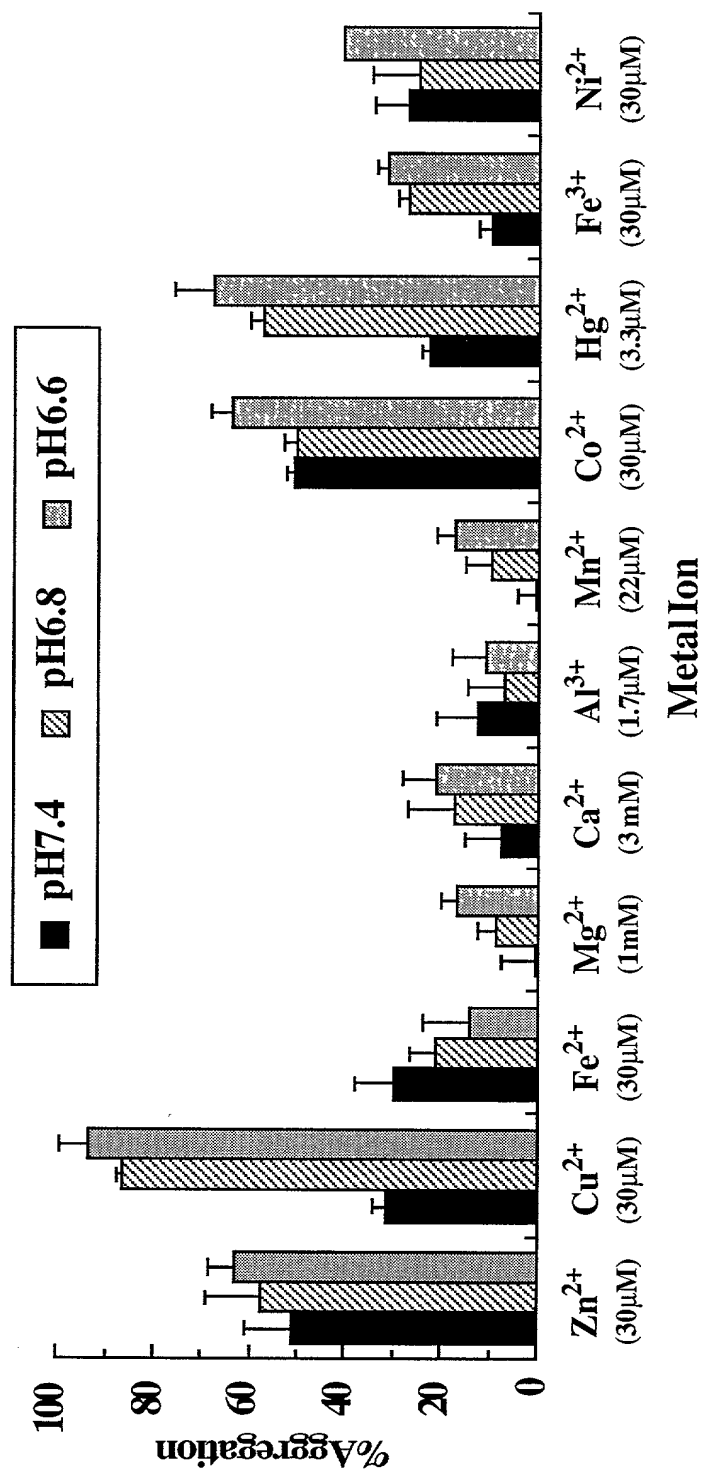


Fig 2c.

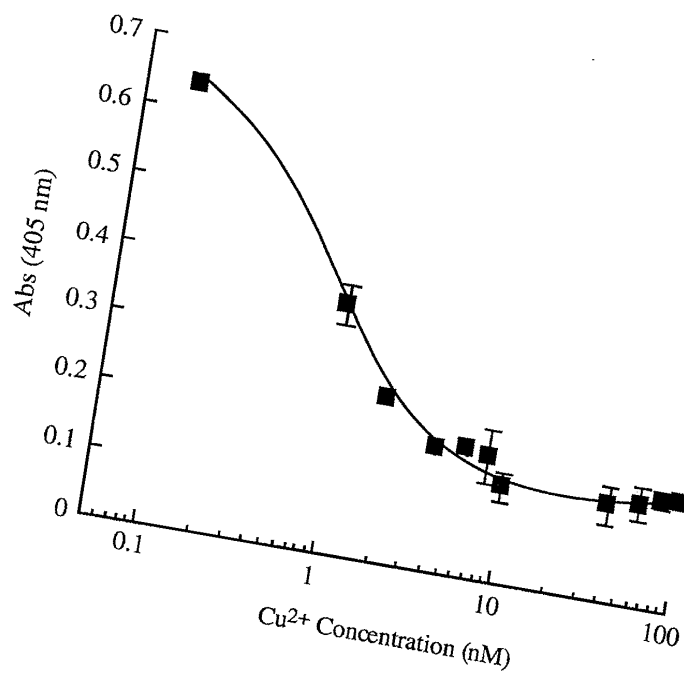


Fig 3

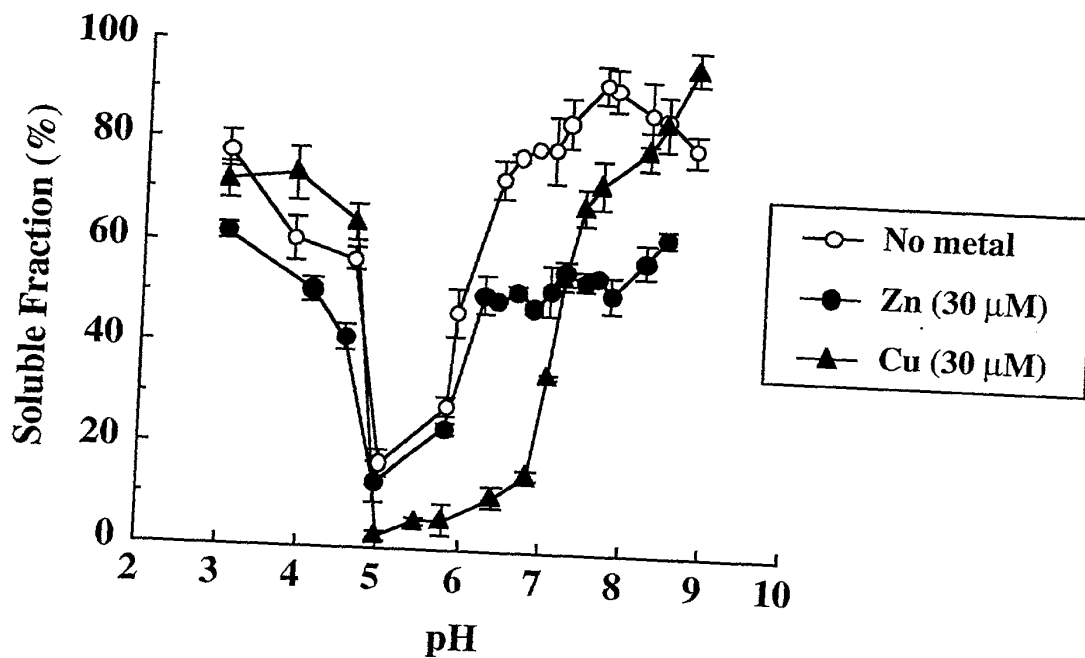


Fig 4a

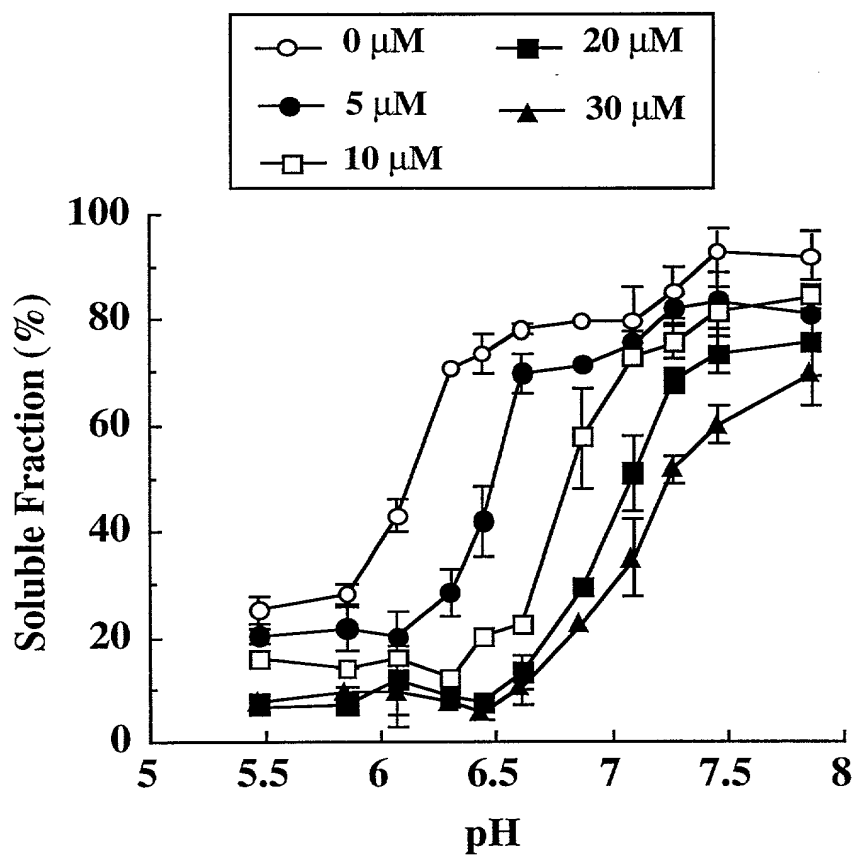


Fig 4b.

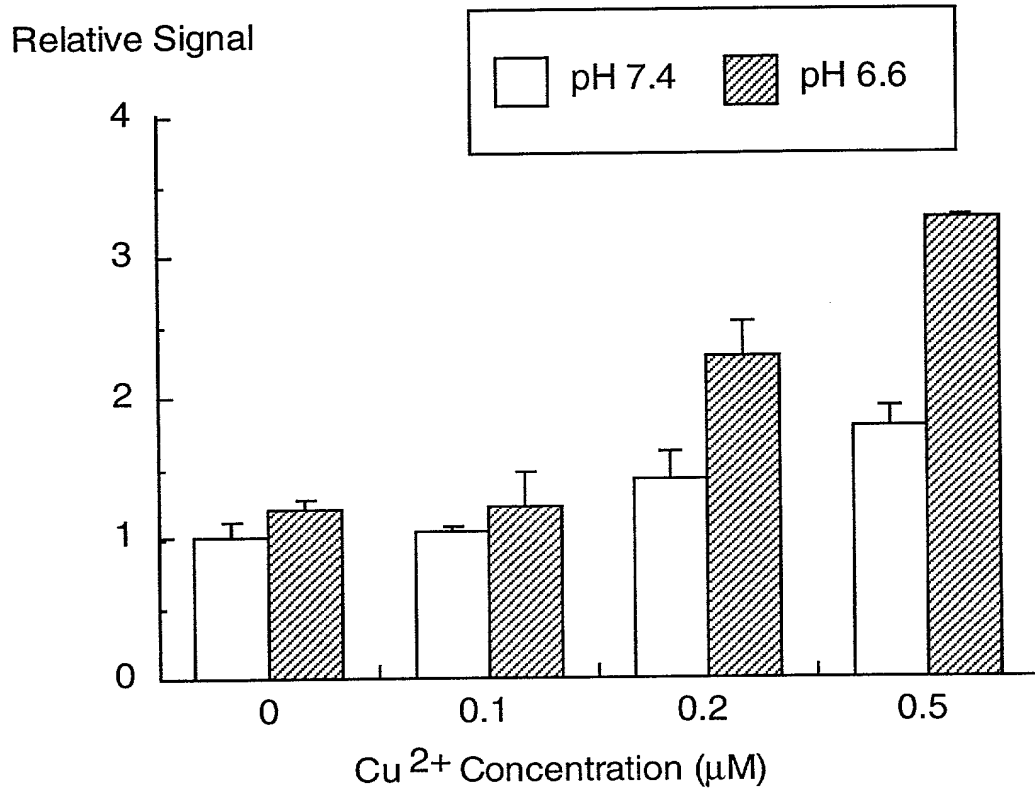
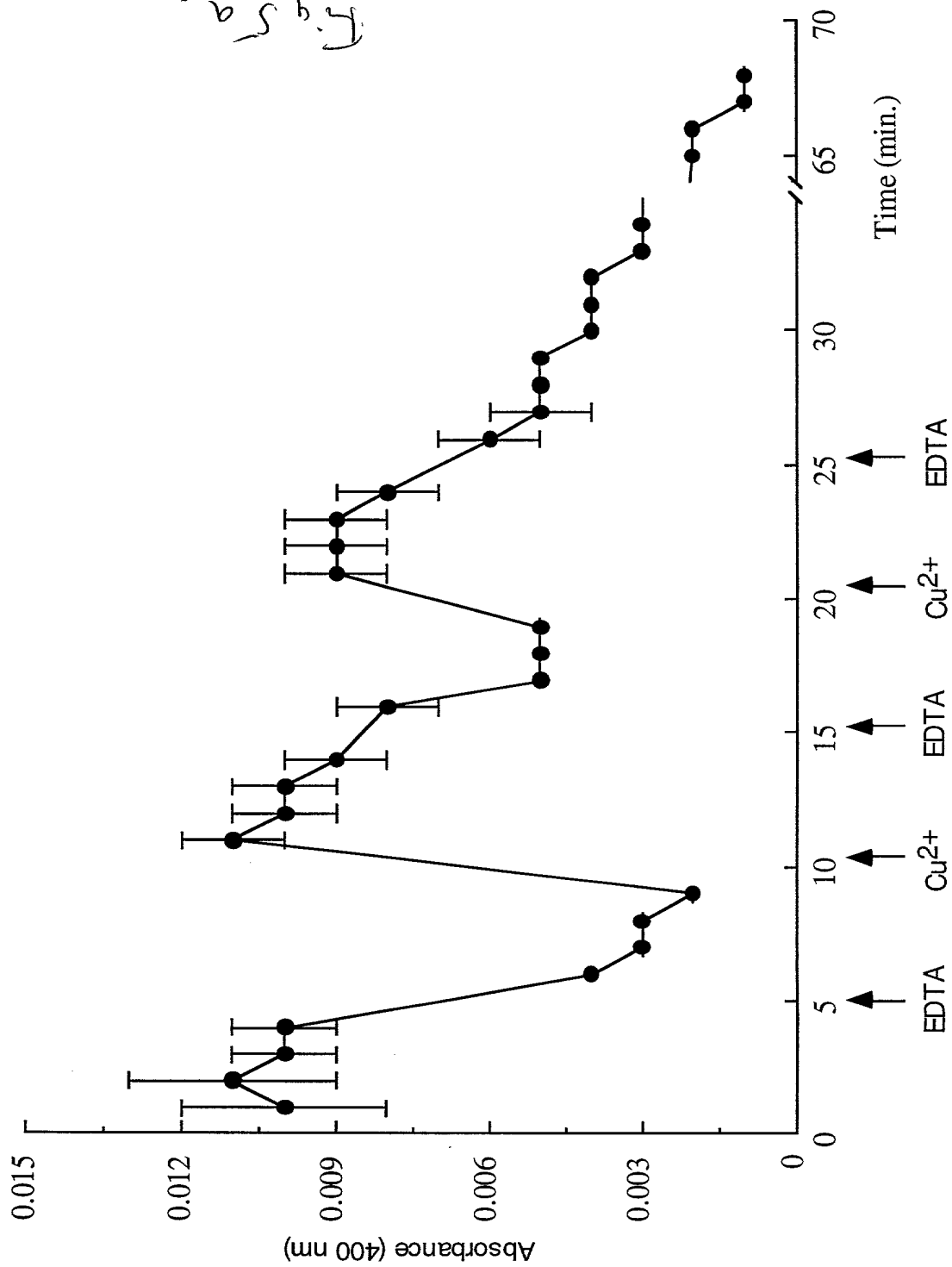


Fig 4 e

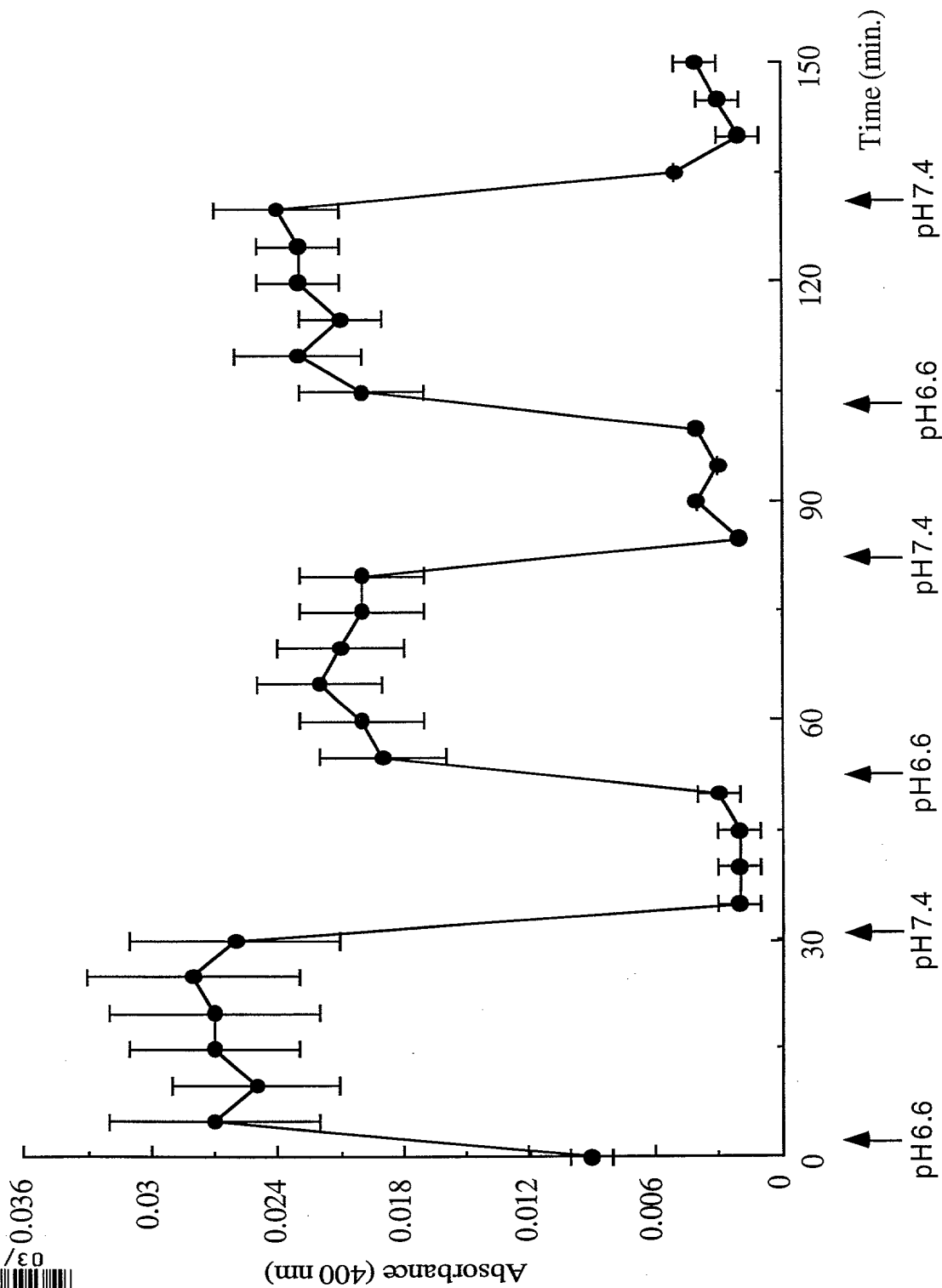
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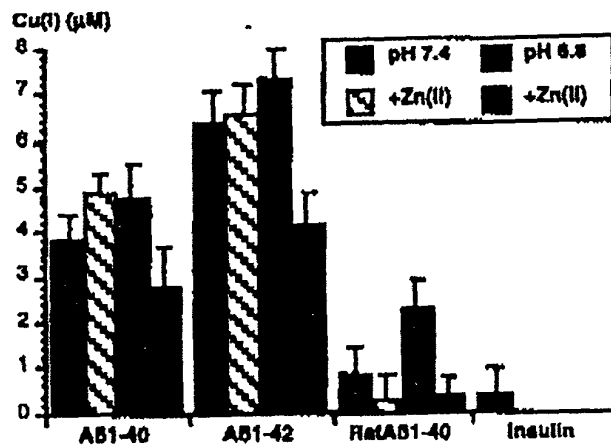


Fig. 6

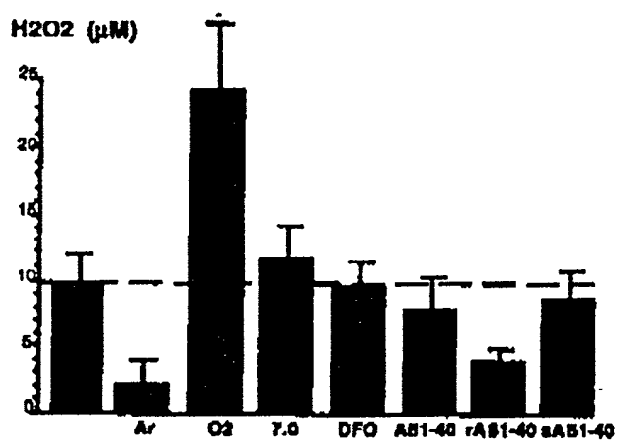


Fig. 7

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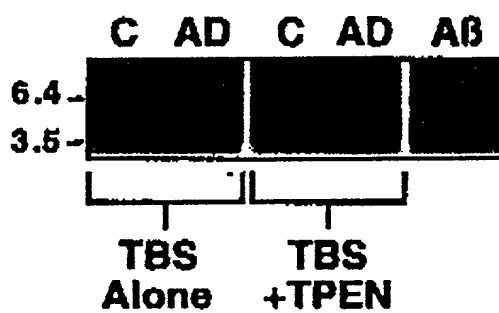


Fig. 8

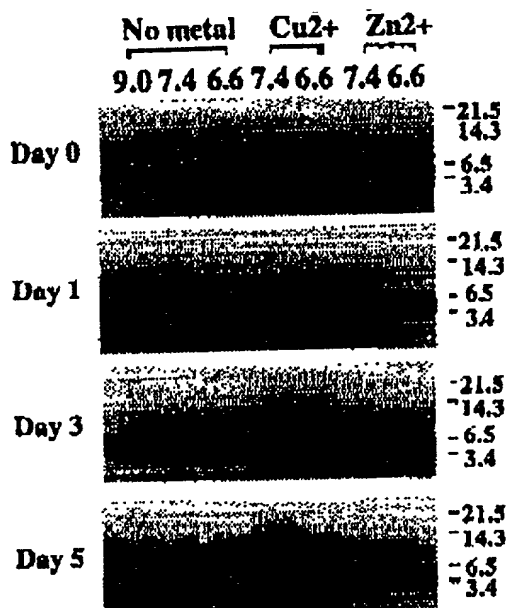


Fig. 9

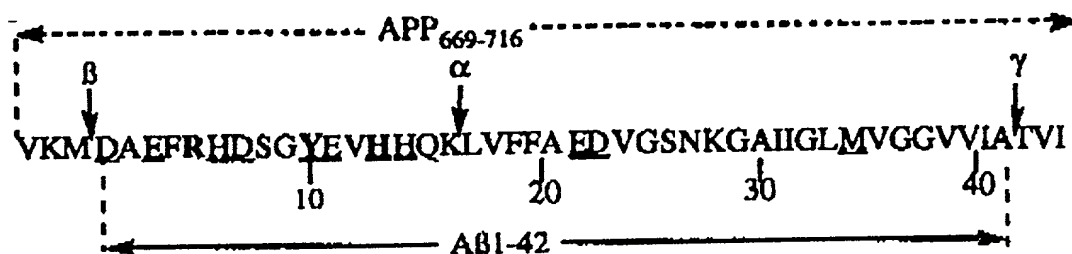


Fig. 10

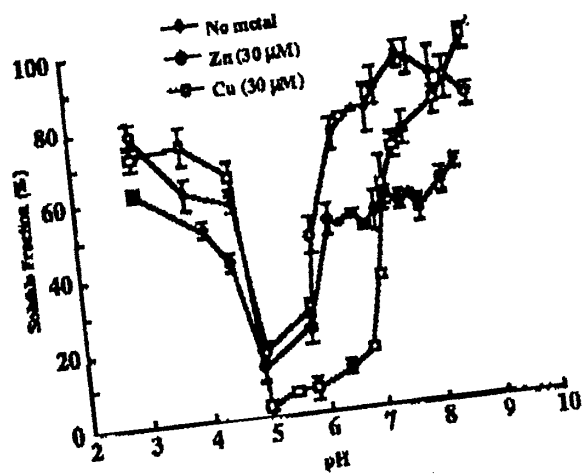


Fig. 11

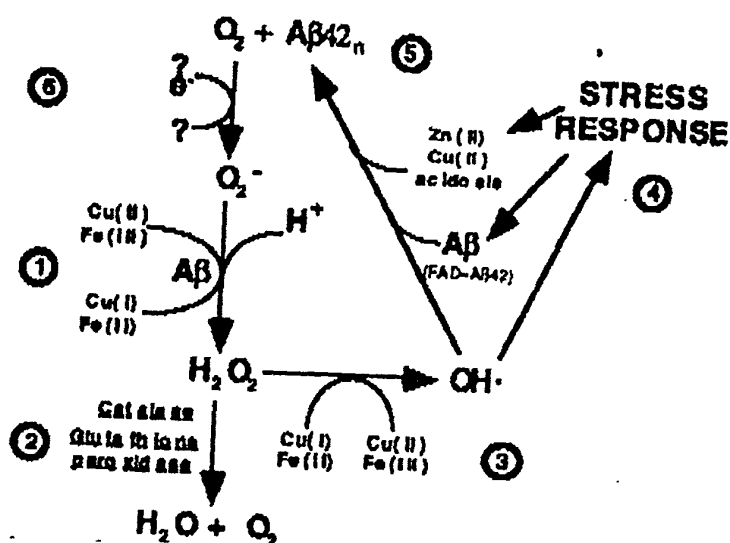


Fig. 12